DNA-driven assembly of micron-sized colloids

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Citation for published version (APA):

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Obtaining temperature reversible colloidal systems

In the first two experimental chapters the aggregation behavior of colloids coated with long and flexible DNA strands was discussed. The two different DNA coatings that, for the sake of brevity, we will refer to as “lambda” and “pBelo”, result in different cluster-sizes, but behave similarly with respect to a temperature change. Heating well above the melting temperature of a 12-base ssDNA overhang, did not lead to redispersion of the clustered colloids. To obtain more structured aggregates, preferably crystalline, the ability to redisperse the colloids upon heating is crucial [35]. In this appendix I describe several approaches that were attempted in order to obtain DNA-colloid systems that would exhibit temperature-reversible behavior. We note that, whereas micron-sized colloids with a dsDNA spacer between the colloid and the 12-base ssDNA overhang remain clustered at all temperatures, systems with the same DNA, grafted to smaller colloids (390 nm) are often redispersable. This suggests that Van der Waals attractions are an important factor to consider, while working with micron-sized colloids. Very long DNA linkers provide an additional attraction that allows the colloids to get sufficiently close for the strong dispersion forces to take over: as a consequence, such colloids will remain clustered upon heating.

A.1 Introduction

Self-assembly of micron-sized colloids with DNA tethers [5, 119] was reported a few years after the first successful DNA-driven assembly of nanoparticles [3]. Whereas all aggregates formed with nanoparticles redispersed upon heating, the redispersion of micron-sized colloids proved much more problematic. Apparently, increasing the size of the colloids plays an important role. In 2005 Crocker and coworkers [6] proposed an explanation. They computed the strength of the interaction between two (complementary) DNA-coated colloids ($U_{dna}$). The depth of this potential is roughly proportional to the radius of the colloid. This means that in a system containing micron sized colloids the interaction between colloids is two orders of magnitude larger than for a similar system of nanoparticles with a size of 10 nm. Without any protective measures, colloids that are drawn to such small separations will undergo irreversible binding due to the Van der Waals dispersion forces acting between the colloids. On the basis of these observations, ref. [6] suggested two methods to obtain reversibility: decreasing $U_{dna}$ or increasing the repulsion between colloids ($U_{rep}$), such that colloids do not get trapped in the Van der Waals interaction.
Waals minimum.

Subsequently, several research groups [6, 7] succeeded in making systems of micron-sized DNA-coated colloids that behaved reversibly with regard to temperature. However, the resulting assemblies tended to be amorphous. The temperature at which experiments are performed, is partly responsible for this finding. For DNA in solution it was found that, while the association step has a relatively small activation energy, the activation energy for dissociation is high (∼6x higher) [172]. The kinetics of hybridization in a DNA-linked colloidal system has not been studied in detail, but it seems likely that similar rates will apply individually to each DNA. It should be added that the overall rate of aggregate formation in a colloidal system is also determined by the diffusion of the colloids. As the dissociation rate of DNA duplexes at room temperature is quite small, the aggregation process occurs more rapidly than rearrangements within the structure due to DNA dehybridization. Clustering is therefore irreversible at room temperature. Allowing for rearrangements is a necessary but not a sufficient condition to obtain ordered structures. In fact, there are several examples of systems of micron-sized DNA-coated colloids that behave reversibly under temperature change [6, 7, 54], but thus far only one such system could be made to crystallize [123].

One of the most important reasons why systems of DNA-coated colloids get trapped in a gel-like state, is the very narrow melting curve of DNA grafted to colloids (Figure A.1). To obtain order within a system the DNA bridging has to be a dynamic, reversible process. The only window of temperatures where this happens is on the narrow melting curve. Crocker and coworkers estimated this window for crystal formation to be only ∼0.5 °C wide [123]. Temperature control is therefore a necessity for crystal formation.

As one of the first research groups to obtain crystal structures with nano-sized particles, Gang and coworkers [34] made an overview of steps necessary to make aggregates with long-ranged order (Figure A.2). After formation of fractal structures, the sample
Figure A.2: In order to obtain crystalline structures colloids need to redisperse above their melting temperature. A crucial step is for the system to spend a period of time around the melting temperature of the DNA used, so that DNA rearrangements are possible. Figure courtesy of Dr. O. Gang [35]

needs to spend some time around its melting temperature to allow for reorganization. Then the system might assemble into a long-ranged ordered crystal after being melted and annealed one last time.

In short, to obtain ordered structures it is vital to control the strength of the attractive forces due to the DNA. As described previously, there are two options to manipulate the interactions between colloids. First, we can reduce $U_{dna}$. Besides reducing the number of hybridizing strands between two neighboring colloids to lower $U_{dna}$, also the number of bases in the “sticky ends” can be adjusted. The second option is increasing (or introducing) the repulsive term $U_{rep}$. This will prevent the colloids from coming too close to each other. Keeping colloids at appropriate distances from each other, colloids cannot get trapped in the Van der Waals minimum. Examples of methods to achieve this include adjusting the length of the DNA, adding a third component to the system to increase the repulsion between colloids or using different colloid sizes.

In addition to controlling the attractive forces due to the DNA, we should also try to eliminate unwanted sources of attraction. One such source of attraction can be the neutravidin that is used to graft the ssDNA segments to the colloids. As shielding the neutravidin on the colloid-surface proved successful before (chapter 4, additional test were done to shield the neutravidin in all systems. It turns out that perfect shielding is virtually impossible to achieve. An alternative is to use covalent grafting of DNA to colloids in order to create a protein-free system.

In this appendix we list all our efforts to obtain systems that are temperature reversible. Although the principle is clear, it proved difficult to make DNA-coated colloids that assemble below the melting temperature of the ssDNA used and redisperse above that temperature. Reducing the size of the colloids increased the number of temperature reversible systems. However, when very long DNA was used, it proved impossible to suppress the attractive forces that keep the colloids from redispersing above the melting temperature of the DNA links.
A.2 Results and Discussion

A.2.1 Reducing DNA length may lead to cluster reversibility.

Chapter 3 described the aggregation behavior of colloids coated with long and flexible DNA. As described in that chapter, this type of colloids aggregated to form finite-sized clusters, surrounded by a DNA “halo”. The resulting clusters were temperature stable. Even heating to 80 °C, i.e. well above the melting temperature of the overhangs (∼ 42 °C), did not redisperse them. The reason why such colloids tend to aggregate can be understood qualitatively on the basis of a theoretical study of Bhatia and Russel [142]. This study showed that a pair of colloids, linked by long, strongly bound telechelic polymers (with a radius of gyration similar to the size of the colloid itself) would attract at short distances under conditions where flat surfaces coated with the same polymers would repel each other. More precisely: what matters is the ratio of the colloid radius to that of the polymer. If that ratio is not much larger than one, the colloids experience a rather strong attraction at short distances. The strength of the attraction scales (to a first approximation) linearly with the number of polymers. Typically, DNA-coated colloids will also repel due to electrostatics. For free colloids coated with long DNA, the electrostatic repulsion plus the steric repulsion provided by the unlinked DNA’s is enough to keep the colloids stable in solution. However, when the complementary “sticky ssDNA ends” on two colloids hybridize, we have a situation similar to the one described by Bhatia and Russel: now a short-ranged attraction is added to the electrostatic repulsion between the colloids. This change from steric repulsion to “steric attraction” may be just enough to allow the colloids to overcome the free-energy barrier that would otherwise protect them from falling into the deep, primary Van der Waals minimum. Whether or not this happens in practice depends not only on the length of the polymers and the radius of the colloids but also on the nature of the DLVO-interaction between the colloids. In this picture, reducing the length of the DNA spacer sufficiently would protect the system against getting stuck in the primary Van der Waals minimum and would make its phase behavior reversible with respect to temperature.\footnote{As both pBelo and λ-DNA are very long polymers, one should perhaps also consider the possibility that these chain molecules induce “bridging flocculation” of the colloids (see, e.g. V.K. La Mer, Discuss. Faraday Soc. 42 248 (1966)). We did not explore this possibility because there is no evidence that the DNA is adsorbed (weakly) to the colloids. However, as La Mer already pointed out, bridging flocculation of colloids - by like-charged poly-electrolytes - is possible.}

If the present simple picture is valid, colloids coated with pBelo-DNA should be more resistant to collapse than colloids coated with λ-DNA. While results did show a slight increase in repulsion between neighboring colloids (shift in position of the first peak of the pair correlation function), these colloids, once clustered, did not redisperse at 80 °C (well above the expected $T_m$ ∼ 42 °C). The third system (no dsDNA spacers) was able to melt back to its initial state (monomers), suggesting that this length of DNA is below the threshold that marks the transition from an irreversible to a reversible behavior.

To test this hypothesis smaller plasmids are needed. To our knowledge there are no other cos-plasmids available. In order to keep the same “sticky-ends”, the ssDNA cos-ends must be added to dsDNA of desired length. In practice this turned out to be
impossible: end-ligation of the cos-overhang to the DNA never results in the desired constructs, as available restriction sites resulted into 4-base overhangs. These overhangs are too small for efficient hybridization of a ssDNA strand containing the cos-sequence. To still test the hypothesis, we use reduced lengths of dsDNA with “sticky-ends” of 4 bases, instead of the 12 base overhangs before.

Two plasmids provide smaller dsDNA spacers (pBR322; 4361 bp and pUC19; 2686 bp). A combination of two restriction enzymes shortened the spacers to 3400 and 2000 bp. It is important to choose an overhang that is not a palindrome, so a distinction can be made between the two populations of colloids (only A-B binding is possible as opposed to A-A or B-B binding). The biotin-label is in this case not added by ligation of oligonucleotides, but built in by an enzyme (klenow exo− polymerase; New England Biolabs) that adds nucleotides (one labeled with a biotin) one by one to any 5’-overhang.

Working with ssDNA overhangs of only 4 bases requires some experimental adjustments. The melting temperature of these short overhangs is below room temperature: ∼12 °C. Indeed, if the colloids are mixed at room temperature and left to aggregate, no sign of cluster formation is seen over a period of two months. In order to study their aggregation behavior we mix them at room temperature, but afterwards place the samples at 4 °C. Reducing the overhang length increases the aggregation time from one day to at least one month. Both sets of DNA-coated colloids aggregated into thin branched percolating clusters (Figure A.3a,b). Despite the further reduced dsDNA length also these systems proved to be temperature stable: increasing the temperature did not redisperse the colloids. Therefore, we decided not to use these DNA linkers.
A. Obtaining temperature reversible...

Figure A.4: Shielding colloidal proteins by varying the DNA length. A: Two sets of colloids were used: pBelo-coated colloids and cos-coated colloids. B: Colloids grafted with a combination of two types of DNA: pBelo and cos-oligonucleotides. Both scale bars indicate 10 µm.

A.2.2 Shielding more neutravidin sites on the colloids is not sufficient for cluster reversibility.

The only DNA-coated colloids that did redisperse upon heating were the cos-coated colloids (no dsDNA spacer). The down side of using these colloids is that we are not able to exploit the polymeric potential of DNA. In chapter 4 we speculate that shielding the neutravidin on the colloids leads to temperature reversible clusters. By using two different sets of DNA-coated colloids (Figure A.4a insert), we shield more neutravidin sites and can still use the polymeric potential of the DNA.

We coat our “A” type colloids with the 12 base oligonucleotide’s (cos 1) and the “B” type colloids with full length pBelo (7500 dsDNA + cos 2). This combination of colloids assembles similar to a system where both colloids are coated with pBelo-DNA (Figure A.4a; chapter 4). Moreover, also these clusters appeared temperature stable.

Another option is to have both sets of colloids coated with a combination of pBelo-DNA (with sticky overhangs) and the same short ssDNA grafted directly to the colloid (Figure A.4b insert). With this method, more neutravidin sites are shielded (compared to pBelo-coated colloids), as the short ssDNA can bind in between alongside pBelo-DNA strands. There is no difference between the clusters obtained with the system of figure A.4a and this system (Figure A.4b). Apparently the extra possibilities to aggregate, due to the cos-oligonucleotides, do not result in different aggregation behavior. Furthermore, this system fails to redisperse as well, when brought to temperatures above the DNAs melting temperature. This could indicate that we did not succeed in shielding all colloidal proteins. Another reason could be that long DNA linkers introduce additional attractive forces [53].
A.2.3 Coating colloids with DNA and a short repulsive brush

To decide whether the inability to redisperse clusters above the melting temperature of the DNA, is indeed an intrinsic property of colloidal systems coated with long DNA, we examined three more systems. Whereas in the previous sections the focus was on shielding the surface, here we describe systems that are coated with DNA as well as a short repulsive brush and thus introduce a repulsive term $U_{\text{rep}}$ to the system.

A polymer-based stabilizer.

Since we use polystyrene beads functionalized with neutravidin, we can graft any third component (to introduce $U_{\text{rep}}$) that has a biotin end to the colloids. First, we tried short biotin labeled polyethylene glycol (PEG; Mw 5000; $\sim$ 30 nm). After coating our colloids with pBelo-DNA we dissolved them in a solution of biotin-PEG (10 mg/ml), allowing the polymers to penetrate the thick adsorbed DNA layer around the colloids. After washing away unbound polymers, the aggregation was examined. The pair correlation function of the fractal aggregates, showed no changes with respect to pBelo-DNA coated colloids (data not shown). Moreover, the structures appeared temperature stable above the melting temperature.

Short blunt dsDNA stabilizers

Adding short biotin-dsDNA with a blunt end to our pBelo-DNA coated colloids leads to colloids that are similar to the ones in the insert in Figure A.4b, except that the blunt end DNA is longer (75 bp; for sequence see materials and methods). The colloids were prepared in the same two-step approach as described above ([dsDNA] 20 $\mu$M).

Aggregating these colloids leads to the same structures as seen earlier with colloids coated with pBelo-DNA without any third component (chapter 4). Although it appeared that these samples needed more time to cluster (twice as long), too few samples where examined to draw definitive conclusions. Moreover, these structures were also irreversible with respect to temperature.

One possible explanation for the failure of achieving temperature reversibility despite the attempt of grafting a steric brush to the colloids is that we did not succeed in making this brush. Although the monolayer of grafted pBelo-DNA around the colloids is rather dilute, the negatively charged backbone of the DNA itself may present a too large Coulomb repulsion for even short biotin-dsDNA to be able to penetrate.

To test if the DNA-coated colloids are grafted with both the DNA and the additional stabilizer (either the PEG-polymer or the short ds-DNA), we repeated the experiments with cos-coated colloids instead of colloids coated with long DNA. As the length of the stabilizer is in both cases longer than the 12 base sticky end ($\sim$ 6 times for both the PEG polymer and the dsDNA), the latter will get buried. We expect this type of colloid to either aggregate with a longer aggregation time or not to cluster at all, as the sticky ends are not easily accessible anymore. Figure A.5c shows a cluster of these colloids. Clearly the clustering is neither prohibited, nor did the aggregation time increase. Both results indicate that we did not succeed in the formation of a sterically stabilizing brush.
Figure A.5: Addition of a third component to the DNA-coated colloids in an attempt to obtain temperature reversibility. A: pBelo-coated colloids with biotin-PEG (Mw 5000) bound to the surface. B: pBelo-coated colloids with a short dsDNA (75 bp) as a third component. C: Both system aggregated also if cos-oligonucleotides were used instead of pBelo DNA, even though the third component is longer than the “sticky-ends” (the picture depicted is with the dsDNA component). All scale bars correspond to 10 µm.

Adding Pluronics to enhance stabilization.

Instead of using biotin to attach additional stabilizing molecules to the colloids by a biotin-neutravidin bond, adsorption is also an option. By using adsorption instead of the biotin-neutravidin bond, we can coat our colloids with a stabilizing agent before we add the DNA. While the DNA is not yet present, the negatively charged backbone of the DNA itself cannot present a large Coulomb repulsion for a third component.

A polymer that can adsorb to the colloids is Pluronics. Pluronics are neutral symmetric triblock copolymers of poly-ethylene oxide – poly-propylene oxide – poly-ethylene oxide. The two outer PEO blocks are soluble in water at all temperatures from 0 to 100 °C, whereas the middle block becomes more hydrophobic with increasing temperature. We work with Pluronics of two different lengths. The longest Pluronic is F108 ((PEO)$_{127}$(PPO)$_{48}$(PEO)$_{127}$ that forms a sterically repulsive brush of $\sim$ 40 nm (calculated). The shorter block copolymer is F68; (PEO)$_{76}$(PPO)$_{29}$(PEO)$_{76}$; it forms a sterically repulsive brush of $\sim$ 20 nm.

Pluronics adsorb to polystyrene colloids by swelling/deswelling-based chemistry [59]. Briefly, an aqueous suspension of polystyrene colloids is mixed with a soluble triblock copolymer. In toluene, the colloids swell and become porous, allowing the triblock’s hydrophobic middle-block to penetrate into the interior. After replacing toluene by an aqueous buffer, the colloids resolidify, trapping the triblock polymers at the surface of their glassy polymer matrix. All neutravidin proteins are still available for DNA binding as no biotin is present in the system.

The short brush made of Pluronics partially prevents hybridization between cos1- and cos2-coated colloids (F68; Figure A.6a), but the long one prohibits it completely (F108; Figure A.6b). Because the cos-experiments looked very promising, we subsequently prepared F108 and pBelo-coated colloids (Figure A.6c). However, even with a repulsive brush, these colloids failed to redisperse at high temperatures.
A.2 Results and Discussion

Figure A.6: Adding Pluronics as a third component fully inhibited the aggregation of cos1 and cos2-coated colloids. Colloids coated with long DNA were not affected. A: F68-stabilized colloids; only small clusters were obtained. B: F108-stabilized colloids; aggregation was prohibited C: pBelo-F108 colloids aggregated as before and did not redisperse upon heating. All scale bars indicate 10 µm.

So far, we showed that there are two factors playing a role in the irreversible binding of the colloids. First, the proteins on the surface of the colloids, second the additional attractive forces exerted by the long DNA. To reduce non-specific attractive forces, we next test a protein free system.

A.2.4 Switching from protein to covalent binding of DNA.

To avoid protein binding, the DNA could be linked to polystyrene colloids covalently. There are multiple ways to connect DNA to a colloids, but we decided on the protocol shown in Figure A.7a, with amino-coated polystyrene beads (diameter 810 nm). First, an ester (succinimidy-[N-maleimidoamino]-dodecaethyleneglycol] ester) is attached to the amino-groups. This ester is not only selected for its maleimide end-group, but also because it has a polymeric midsection that can give additional stabilization. The presence of maleimide groups on our colloids can be tested with help of a Fmoc test [173]. Fmoc-cysteine is a molecule, that can be bound to the maleimide with the thiol-group of the cysteine (Figure A.7b). Adding a base (piperidine) will release the Fmoc, which becomes a fluorophore when in solution, that can be detected quantitatively. Liquid chromatography showed that ~ 60% of all amino groups reacted with the added ester. Since the ester is present on the colloids in sufficient amounts, the maleimide colloids are coated with thiol-labeled DNA (prepared similar to biotin-DNA).

The resulting colloids aggregate into a fine stranded network (Figure A.8a). Even though the ester used is specifically selected for its polymeric character, heating up to 80 °C did not redisperse the colloids. In an attempt to stabilize the colloids further, Pluronics is added. This had the reverse effect: colloids with Pluronics aggregated within seconds in the absence of DNA (Figure A.8b). As additional stabilization proved to be impossible and the preparation of these colloids took more time than the protein binding we decided to abandon this route.

The experiments with DNA linked covalently to the colloids show, that by mere
Figure A.7: Overview of molecules and reaction steps used to obtain colloids with covalently bound DNA and of those used to determine the yield. A: Reaction steps used to bind DNA covalently to PS-colloids functionalized with amino-groups. B: To determine the yield of reaction step 2, a Fmoc test was performed (see text).
shielding of the proteins, we will most likely never obtain a micron-sized colloidal system (coated with long DNA) that is temperature reversible.

A.2.5 Additional stabilization of cos-coated colloids to reduce the melting temperature.

At this point, the only colloids that redispersed are the cos-coated colloids. Even though these colloids can switch between a bound and an unbound state, the temperature at which this takes place (80 °C), is higher than the expected melting temperature of DNA of this length (42 °C for the cos1/cos2 pair). We decided to introduce a short double stranded DNA spacer with 50 bases dsDNA between the biotin and the “sticky ends” (sequence in materials and methods). Addition of the spacer should keep the colloids further apart, thereby lowering attractive surface forces and thus the melting temperature.

As expected, these colloids displayed the same aggregation behavior as the cos-coated colloids (Figure A.9a), but instead of lowering the melting temperature, the opposite happened. It was impossible to redisperse these clustered colloids within the accessible temperature window (up to 80 °C). This shift toward inaccessible melting temperatures may be understood in terms of the possible number of hybridization sites in the contact area. By introducing the double stranded DNA spacer we increase the configurational entropy of the cos-ends and therefore also the number of successful bonds between opposing cos1/cos2 colloids [6].

As the grafting density of “sticky ends” plays a role in temperature reversibility [6], we varied the number of “sticky ends” per colloid. A mixture of biotin-dsDNA of 50 bp with and without the 12 base cos-overhangs is grafted to the colloids. Colloids displayed 10 to 90% “sticky ends”. Still, no reduction in the melting temperature was found.

Figure A.8: Colloids with covalently bound DNA. A: Aggregation of colloids with DNA covalently bound to them (the DNA used was pBelo). B: Additional components made these colloids highly unstable; aggregation possible without DNA present. Both scale bars correspond to 10 µm.
Finally, we repeated the experiments with the colloids also coated with 50 bp dsDNA, but displaying shorter “sticky ends” (8 bases). These eight bases are expected to melt around 30°C, allowing us to work at room temperature. Again, similar aggregates were found, but once formed no melting could be observed (Figure A.9b).

### A.2.6 Smaller colloids have lower Van der Waals forces.

From the previous paragraphs it appears that non-specific interactions play an important role in our systems. In order to circumvent this problem, we tried smaller colloids, as smaller colloids tend to exert weaker Van der Waals attractions [174]:

$$U_{vdw}(r) = -(A/6d)(r/2)$$

Where $A$ is the Hamaker constant, $d$ the distance between two colloids and $r$ the radius of the equal sized colloids. It should be stressed that this expression is only valid in the limit $d << r$ - but that is precisely the region that dominates the “collapse” due to dispersion forces.

### Unequal colloid sizes.

For practical reasons, we started with colloids of unequal sizes. The smaller colloids we chose to work with (neutravidin-coated polystyrene; diameter 390 nm) were only available in one fluorescent color. The other color had to be custom made (Bangslab).

Most DNA constructs were tested with the smaller green-fluorescent colloids (cos; 50 bp dsDNA + cos ssDNA; 50 bp dsDNA + 8 bases ssDNA and pBelo-DNA). All DNA constructs showed similar aggregation behavior. An example is depicted in Figure A.10a. Reducing the colloid size of the green fluorescent colloids, while keeping the large red fluorescent colloids, resulted in more systems that redisperse upon heating. In contrast to the behavior observed before, now both the systems with the 50 bp dsDNA spacer melted above 80 °C (Figure A.10b). As reducing the size of half the colloids used,
Figure A.10: The Van der Waals attractions between smaller colloids are smaller than for larger ones. A: Unequal bead sizes; combination of 1 µm colloids and 390 nm colloids (50 bp dsDNA + 12 ssDNA). B: By reducing the size of half of all colloids, the samples with 50 bp dsDNA redisperse upon heating. C: Aggregate of 390 nm DNA-coated colloids (50 bp dsDNA + 12 ssDNA). D: Again, the samples with 50 bp dsDNA redisperse upon heating. Scale bars correspond to 10 µm (A, B) or 5 µm (C, D).
Figure A.11: Smaller colloids are harder to image due to their size, but mainly due to fluorescence interference. A: In time, red-fluorescent unidentified objects start to interfere with imaging (for clarity only the red-fluorescent colloids are shown (in gray)). B: Image with the same unidentified objects out of focus to show the extent of interference. Scale bar measures 5 µm.

resulted in two more DNA-coated systems, it seems that Van der Waals forces indeed prevent bigger colloids from redispersing. But Van der Waals attractions are not the only factor responsible as the colloids coated with pBelo-DNA remained clustered at 80 °C.

Nano-sized colloids.

Smaller colloids (diameter 390 nm; both colors) were tested with the same set of DNA (Figure A.10c). While all assemblies of the DNA-coated colloids are again similar, differences in melting temperature are detected. Starting at 50 °C and increasing the temperature with steps of 5 °C/2 hrs, showed that the 8 bases “sticky ends”-system melted first at ~ 60 °C. The colloids coated with the same 50 bp dsDNA spacer, but with a longer overhang (12 bases) melted at a higher temperature: 65 °C (Figure A.10d). Cos-coated colloids melted at ~ 70 °C indicating that a short dsDNA spacer indeed keeps colloids further apart. All melted samples are able to reform (cluster again) when brought back to room temperature, indicating that it is indeed due to the melting of the DNA-overhangs (data not shown). The colloids coated with pBelo-DNA remained clustered. With all other samples dispersing, this clustering cannot be due to the size of the colloids alone. Instead, the longer DNA has to play a role in this effect.

Next, we test if we can increase the order within the aggregates by following the work of Gang and coworkers (see Figure A.2, [34]). Briefly, their research stresses the importance of temperature cycles where after formation of fractal structures, the sample needs to spend some time around the melting temperature of the DNA to allow for reorganization. Then the system might assemble into a long-ranged ordered crystal after being melted and annealed one last time.
Colloids coated with 50 bp dsDNA + 8 bases ssDNA are mixed at room temperature and left to aggregate. Then, the sample is placed at the melting temperature of $\sim 60 ^\circ C$ (Figure A.12d). (PCR-apparatus; eppendorf mastercycler). The PCR apparatus was used to slowly and controllably cool down the sample ($1 ^\circ C/min$), allowing rearrangements of the DNA. As a control we also added a sample containing “bare” colloids. Unfortunately, the “bare” neutravidin colloids were not stable under the experimental conditions (Figure A.12a), as small aggregates floated in the solution. Although, the sample with DNA-coated colloids (50 bp dsDNA + 8 bases ssDNA) showed different clustering (Figure A.12b) from a sample left at room temperature (Figure A.12c), we did not see an increase in order. The continued lack of order is not a real surprise as Gang and coworkers [34] concluded that DNA needs to be flexible in order for colloids to crystallize and the DNA used in this experiment is rigid.

It would be interesting to test longer DNA constructs (but shorter than pBelo-DNA, see future directions) on this size of colloids. But before these experiments can commence, a minor problem needs to be addressed: the red-fluorescent colloids leak some of their fluorophore in time. After a few days, strongly fluorescent spots prevent proper imaging of the aggregates (Figure A.11a,b). Peculiarly, these fluorescent spots are only detected in samples containing DNA-coated colloids. Colloids without DNA did not leak any fluorophores under identical circumstances. We did try changing the buffer conditions, but did not succeed in preventing the fluorophore from leaking. Additional experiments are needed before the system is optimized to test different spacers lengths on the smaller colloids.

A.3 Discussion and Outlook

Developing a system of DNA-coated colloids that can assemble or disassemble depending on the temperature of the system, turned out to be very difficult. Theory arguments provided us with a couple of options to try. The results above show that the reality of laboratory experiments can be so complex that simple theoretical arguments may fail.

The few systems that we were able to make temperature-reversible all had short DNA strands (see Table A.1 for an overview). As there are, to my knowledge, at this stage no other experiments on colloids coated with very long, flexible DNA spacers in combination with micron-sized colloids, we cannot compare our results directly to other findings. Our experiments suggest that the long DNA that we use pushes the colloids close together, into a regime where the Van der Waals interactions are too strong to allow subsequent redispersion. Micron-sized colloids coated with short DNA strands have been used by several other groups. However, the systems are slightly different. For instance, the 50 bp dsDNA + 12 bases ssDNA system that we studied did not melt at any temperature. In contrast, Chaikin and coworkers [62] found a melting temperature of 42 $^\circ C$ for a system with 49 bp dsDNA + 11 bases ssDNA. Dreyfus et al. did however use a different type of colloid (Dyna-magnetic colloids) - as Dyna-magnetic colloids are also polystyrene based, the difference must be due to the (unknown) details of the chemical synthesis of the colloids.
Figure A.12: Starting the assembly process above the melting temperature does not lead to an increase in order. A: “Bare” colloids (no DNA) are not stable under the experimental conditions. B: DNA-coated colloids (50 bp dsDNA + 8 bases ssDNA) that were assembled above the melting temperature. Although the assemblies look different than before, we think this is due to the removal from the PCR apparatus rather than a temperature effect. C: Control sample, mixed and left at room temperature to aggregate. D: Control sample, mixed and left at \( \sim 50 \) °C to aggregate. All scale bars indicate 5 \( \mu m \).
Figure A.13: Schematic representation of the singlet fraction in a colloidal system with respect to time and temperature. A: DNA-coated colloids follow a sharp melting transition. As rearrangements of the DNA are crucial for obtaining order, there is only a small working window with this type of colloids (see gray shaded area). B: The latest system of DNA-coated colloids of Chaikin and coworkers [175], changes the steepness of the curve by using DNA-coated colloids, with DNA that can form hairpin structures and intra-particle DNA bridges. Now the area where DNA rearrangements can occur, is increased significantly (compare shaded areas from A and B).

The steepness of the melting curve for DNA-coated colloids remains a problem. Positioning mistakes that occur during this aggregation process, immediately cause colloids to bind in the wrong place as there is no time for rearrangements (Figure A.13a). A recent paper [175] described a procedure to change the slope (and path) of this temperature curve by using DNA-coated colloids that could not only hybridize to each other, but were also able to form hairpin structures with their “sticky ends”. Hairpin formation leads to self-protected (inert) overhangs. Starting at high temperatures, these colloids first get prepared in a state where most colloids are relatively inert monomers - these monomers are then arranged into the desired pattern and are then made to hybridize with their neighbors by increasing the temperature, such that the hairpins unfold and the inter-colloid DNA-links can form (Figure A.13b).

Crocker and coworkers also pointed out the importance of the type of colloid used. With a depletion study of four different types of colloid against small carboxylated colloids they discovered differences in aggregation behavior. Whereas the big carboxylated colloids and their homemade PEGylated spheres formed close-packed crystal structures under depletion (Figure A.14a), the neutravidin- and glycine modified colloids rather formed large aggregates (Figure A.14b). This supports the suggestion that the failure to crystallize our temperature reversible systems might not be an effect of the DNA alone. Rather, the colloidal surface chemistry may affect the ability of these colloids to anneal into ordered structures being dependent on the colloids surface chemistry. However, as chapter (chapter 5) showed, colloids coated with long DNA can form crystal structures, be it under rather different conditions.
Table A.1: Overview of all DNA-coated colloidal systems studied. If a system is temperature reversible, the melting temperature is listed. A “X” indicates that once aggregates formed with that type of DNA-coated colloids, the system could not be redispersed at any temperature.

<table>
<thead>
<tr>
<th>colloid sizes</th>
<th>DNA-construct</th>
<th>$T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 1 µm; B: 1 µm</td>
<td>λ-DNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>pBelo-DNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>pBR322-DNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>pUC19-DNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>50 bp dsDNA + 12 ssDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>50 bp dsDNA + 8 ssDNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>cos (12 ssDNA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pBelo + cos (12 ssDNA)</td>
<td>80 °C</td>
</tr>
<tr>
<td>A: 390 nm; B: 1 µm</td>
<td>pBelo-DNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>50 bp dsDNA + 12 ssDNA</td>
<td>80 °C</td>
</tr>
<tr>
<td></td>
<td>50 bp dsDNA + 8 ssDNA</td>
<td>80 °C</td>
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<tr>
<td></td>
<td>cos (12 ssDNA)</td>
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<tr>
<td></td>
<td>pBelo + cos (12 ssDNA)</td>
<td>X</td>
</tr>
<tr>
<td>A: 390 nm; B: 390 nm</td>
<td>pBelo-DNA</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>50 bp dsDNA + 12 ssDNA</td>
<td>65 °C</td>
</tr>
<tr>
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<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>pBelo + cos (12 ssDNA)</td>
<td>X</td>
</tr>
</tbody>
</table>

A.4 Future directions

It would be interesting to test longer DNA constructs (but smaller than pBelo-DNA) on colloids of 390 nm. At the moment, a system of colloids coated with dsDNA of 50 base-pairs and a 12 base “sticky ends” is temperature-reversible, whereas the use of a pBelo spacer (7500 bp) leaves a system irreversible with respect to temperature. As there is more than two orders of magnitude difference in the length of these two types of DNA strands, there is a clear need for a systematic study of the self assembly of colloids coated with DNA of intermediate length. In this appendix two more lengths were already examined, but it seems preferable to clone the cos-overhang into plasmids of different sizes (Figure A.15a). With such an approach the dsDNA spacer can be varied at will as plasmids are available in a broad size range (1000 to 1000.000 bp) and restriction enzymes can shorten plasmids to the exact desired length, while the ssDNA overhang (attractive part) will be the same for all colloids studied (in contrast to the 4-base overhangs used before).
A.4 Future directions

Both experimental papers that reported the observation of three-dimensional crystals [34, 35] stress the importance of flexible spacers. This is another reason why a systematic study of intermediate spacer lengths would be definitely worthwhile.

If the steepness of the melting curve remains to be a problem, it may be worthwhile to consider the use of colloids coated with temperature-switchable DNA hairpins for the formation of ordered structures [175]. In order to be able to clone these in plasmids of different sizes, one would have to order two hairpin sequences extended on one side with a sequence of DNA with a known restriction site within the plasmid of choice (Figure A.15b), together with two complementary DNA strands to the sequence before the hairpin. Before cloning, the two hairpin sequences need to be hybridized together with the two complementary sequences of the restriction sites. This will result in a piece of DNA with two nicks into the backbone, see Figure A.15b. As cloning goes best if two different restriction enzymes are used, the extensions on the hairpin DNA sequences cannot be the same (indicated with two scissors of different color). Two different restriction sites work best, as then the receiving plasmid cannot close without uptake of the donor DNA strand. Note that the resulting hairpin-plasmid is like a λ-DNA vector: it can open upon heating as the backbone is not intact, while the new cosmid has to be cleaved just like the pBelo-plasmid. If this last cloning method would not yield the desired result, one can consider using nicking enzymes instead. Then the extended sequence should contain a nicking recognition site as well as a restriction site.

By varying the length of DNA one can examine if one could also self assemble micron-sized colloids into a three dimensional crystal, opening routes to test if all crystal-structures predicted theoretically can also be realized experimentally.
Figure A.15: Cloning strategies to obtain different dsDNA spacer lengths. A: New cosmid-plasmids can be obtained by cloning the cos-site sequence of pBelo into any plasmid of choice. B: Obtaining dsDNA ended by a hairpin ssDNA sequence could in principle also be realized with cloning techniques. This protocol could be more troublesome due to the non-continuous backbone of the plasmid.
A.5 Materials and Methods

A.5.1 Preparation of biotin-DNA

pBelo-DNA

The pBeloBac11 plasmid was purchased as a strain (New England Biolabs, ER2420S). To obtain sufficient DNA for coating 50 µl colloids (1% solids), the strain was grown overnight at 37 °C in 60 ml LB medium (for 100 ml: Bacto-Tryptone 1 g; Bacto-Yeast extract 0.5 g; NaCl 1 g and ddH2O to 100ml; autoclave to sterilize) in the 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. The plasmid contains a cos-site that can be opened by restriction with λ-terminase (BIOzymTC), leaving two 12 base single strands (“sticky ends”). The linearized DNA was separated into two batches. One batch was mixed with a solution (5 µl; 20 µM) of cos1-biotin oligonucleotides (Eurogentec), the other with cos2-biotin oligonucleotides (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. 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 (Millipore) with Tris-HCl buffer (250 mM, pH = 8). The biotin-DNA solution was then recovered in a clean tube.

pBR322-DNA

The pBR322 plasmid was used to obtain shorter length DNA. To obtain dsDNA with complementary ssDNA “sticky ends”, two preparation methods were used. For our “A”-type colloids, the circular plasmid (New England Biolabs) was first restricted with HindIII (New England Biolabs), leaving two 4-base ssDNA ends. Both ends were then filled up nucleotide by nucleotide with Klenowexo⁻. (To obtain biotinylated DNA regular dTTP, dATP, dGTP-nucleotides were used in combination with biotin-dCTP; all Invitrogen). The reaction solution was then cleaned from excess nucleotides and enzyme (PCR-purification; Qiagen). The obtained DNA was further restricted with BsaI (New England Biolabs) to obtain a 4-base “sticky end” (5'-CGGT-3'). To remove excess enzyme and the shortest end of the restricted DNA, again the DNA was cleaned (PCR-purification-kit; Qiagen) and recovered in a clean tube. For our “B”-type colloids, the circular plasmid (New England Biolabs) was first restricted with AflIII (New England Biolabs), leaving two 4-base ssDNA ends. Both ends were then filled up nucleotide by nucleotide with Klenowexo⁻. (To obtain biotinylated DNA regular dTTP, dATP, dGTP-nucleotides were used in combination with biotin-dCTP; all Invitrogen). The reaction solution was then cleaned from excess nucleotides and enzymes (PCR-purification; Qiagen). The DNA was then also restricted with BsaI (New England Biolabs) to obtain a 4-base “sticky end” (5'-ACCG-3'). To remove excess enzyme and the short end of the restricted DNA, again the DNA was cleaned (PCR-purification-kit; Qiagen) and recovered in a clean tube. Both sets of enzymes were chosen to obtain dsDNA of almost the same length (3398 bp vs 3407 bp) with complementary ssDNA “sticky ends”.
pUC19-DNA

Besides pBR322, another plasmid was used to further reduce the dsDNA length. Again two preparation methods were needed to obtain dsDNA with complementary ssDNA “sticky ends”. Here, for our “A”-type colloids, the circular plasmid (New England Biolabs) was first restricted with HindIII (New England Biolabs), leaving two 4-base ssDNA ends. Both ends were then filled up nucleotide by nucleotide with Klenow exo⁻. (To obtain biotinylated DNA regular dTTP, dATP, dGTP-nucleotides were used in combination with biotin-dCTP; all Invitrogen). The reaction solution was then cleaned from excess nucleotides and enzymes (PCR-purification-kit; Qiagen). The obtained piece of DNA was further restricted with BseYI (New England Biolabs) to obtain a 4-base “sticky end” (5’-CTGG-3’). To remove excess enzyme and the sort piece of restricted DNA, again the DNA was cleaned (PCR-purification-kit; Qiagen) and recovered in a clean tube. For our “B”-type colloids, the circular plasmid (New England Biolabs) was first restricted with BsaI (New England Biolabs), leaving two 4-base ssDNA ends. Both ends were then filled up nucleotide by nucleotide with Klenow exo⁻. (To obtain biotinylated DNA regular dTTP, dATP, dGTP-nucleotides were used in combination with biotin-dCTP; all Invitrogen). The reaction solution was then cleaned from excess nucleotides and enzymes (PCR-purification; Qiagen). This piece of DNA was then also restricted with BseYI (New England Biolabs) to obtain a 4-base “sticky end” (5’-CCAG-3’). To remove excess enzyme and the sort piece of restricted DNA, again the DNA was cleaned (PCR-purification-kit; Qiagen) and recovered in a clean tube. Both sets of enzymes were chosen to obtain dsDNA pieces of almost the same length (2023 bp vs 2036 bp) with complementary ssDNA “sticky ends”.

50 bp dsDNA + 12 base overhang (ssDNA)

This length of DNA does not require the restriction of a plasmid. Instead, needed sequences can be ordered on demand (Eurogentec). To obtain this type of DNA three ssDNA sequences were ordered. First a 50 base ssDNA piece of DNA labeled with a biotin-tag on its 3’-end. (seq1: 5’-GGGTT TTAAG CTTAC CATGG GATAT CCCTA TGATG TGCCA GACTA CGCGG-3’). Then two pieces of 62 bases ssDNA, containing the complementary 50 bases from sequence one plus either cos1 or cos2 (12 bases). (seq2 (cos1): 5’-CCGCG TAGTC TGCCA CATCA TAGGG ATATC CCATG GTAAG CTTAA AACCC GGGCG GCGAC CT-3’; seq3 (cos2): 5’-CCGCG TAGTC TGCCA CATCA TAGGG ATATC CCATG GTAAG CTTAA AACCC AGGTC GCCGC CC-3’). The DNA construct was prepared by mixing equimolar solutions (20 µM; Tris HCl-buffer, 100mM, pH = 8) of either sequence 1 and 2 or sequence 1 and 3. The mixed solutions were heated to 65 °C for ~30 minutes and then cooled overnight to room temperature, resulting in 50 bp dsDNA with complementary 12 base ssDNA “sticky ends”.

50 bp dsDNA + 8 base overhang (ssDNA)

This type of DNA was prepared according to the same protocol as described above. To obtain 8 base overhangs we simply ordered the last 8 bases of the cos-sequences. This
resulted in the following sequences: seq1: 5'-GGGTT TTAAG CTTAC CATGG GATAT CCCTA TGATG TGCCA GACTA CGCGG-3', seq2 (shortened cos1): 5'-CCCGG TAGTC TGGCA CATCA TAGGG ATATC CCATG GTAAG CTTAA AACCC CGCGG CCC-3', seq3 (shortened cos2): 5'-CCCGG TAGTC TGGCA CATCA TAGGG ATATC CCATG GTAAG CTTAA AACCC CGCGG CCC-3'.

A.5.2 Preparation of thiol-DNA

To obtain thiol-labeled pBelo-DNA the same protocol was used as to obtain biotin-labeled pBelo-DNA. Instead of mixing the two batches with cos-biotin oligonucleotides, cos-thiol oligonucleotides were used (Eurogentec; 5 µl; 20 µM). As a disulfide group protects the more reactive thiol-group, the oligonucleotides have to be cleaved to be able to react with colloids.

To cleave of the protective group, a solution of tris(2-carboxyethyl)phosphine (TCEP; 10 mM final concentration) was added to thiol-pBelo DNA. This solution was allowed to react overnight at 4 °C. The next day the solution was cleaned with a PCR-purification-kit (Qiagen) to remove excess TCEP and the protective group. The reaction described is sensitive to air, so all solutions added were first degassed (N₂). The thiol-DNA was collected in a clean tube, and used directly.

A.5.3 Preparation of DNA-coated colloids

protein binding

As in Ref. [53] we used a binary system in which DNA mediated attractions are favorable only between heterogeneous colloids. Green and red fluorescent neutravidin-coated polystyrene micro-spheres (diameter 1 µm, Molecular Probes) were functionalized with DNA carrying complementary “sticky ends”.

Our conjugation protocol is based on the neutravidin-biotin coupling procedure described in Ref. [53]: the batches of DNA were separately mixed with either green (“A” hereafter) or red (“B” hereafter) fluorescently labeled colloids, coated with neutravidin, that were dispersed in TRIS-HCl (250 mM, pH = 8). In each case the DNA and colloids were reacted overnight during which they were continuously tumbled. The next day the samples were pelleted and washed five times to remove excess non-conjugated DNA. Between these washing steps, samples were heated once for 10 min. at 50 °C, to remove poorly bound DNA.

All types of DNA-coated colloids were diluted in a fresh TRIS-HCl buffer (250 mM final concentration, pH = 8) with D₂O to minimize sedimentation.

covalent binding

In order to covalently bind DNA to colloids, we ordered amino-coated polystyrene colloids (diameter 800 nm, Microparticles GmbH). These colloids were first coated with an ester (Pierce; succinimidyl-[(N-maleimidopropionamido)-dodecaethyleneglycol]). This ester binds to the amino groups of the colloids and displays a maleimide end group to
later link thiol-labeled DNA to. An overview of the reaction steps is given in Figure A.7a.

To start coating colloids with the ester, 25 µl (1%) of both types of colloids were washed and redispersed in 50 µl dimethylsulfoxide (DMSO). To this, 20 µl of 250 mM ester-solution (DMSO; Sigma-Aldrich) was added. The reaction was allowed to proceed overnight at room temperature. The next day the excess of ester was removed by pelleting the colloids and five subsequent washing steps with DMSO. Colloids were redispersed in 25 µl DMSO and stored at 4 °C.

The yield of this reaction can be tested by performing a Fmoc test (Figure A.7b). To this end, 10 mg of Fmoc-cysteine (Chem-Impex) in 50 µl DMSO is added to 25 µl ester-coated colloids. The reaction is allowed to proceed overnight at 4 °C. The next day, the excess of Fmoc is removed by pelleting the colloids and washing them five times (DMSO). After removal of the supernatant, the colloids are dried by a Nitrogen airflow. It is important to weigh the dried colloids to obtain the yield of the reaction. To the dried colloids 100 µl 20% piperidine (Sigma-Aldrich) in Dimethylformamide (DMF; Sigma-Aldrich) is added. Immediately afterwards, 900 µl Tetrahydrofuran (THF; Sigma-Aldrich) is added and the sample is mixed. After 15 minutes, the absorbance can be measured at 277 nm. By comparing the obtained value to a calibration curve of reacted Fmoc-cysteine, the yield can be determined. For the above protocol ~ 60% of all amino groups reacted with the added ester. In practice this means the colloids are coated with ~ 1.5 10⁶ reactive groups. As this is far more then DNA strands can be added, no steps were taken to improve the protocol.

The maleimide modified colloids were mixed with thiol-labeled DNA. The mixture was left overnight (at 37 °C) during which they were continuously tumbled. The next day the samples were pelleted and washed five times (TRIS; 250 mM) to remove the excess of non-reacted DNA. Then DNA-coated colloids were diluted in a fresh TRIS-HCl buffer (250 mM final concentration, pH = 8) with D₂O to minimize sedimentation.

### A.5.4 Preparation and coating imaging chambers

To avoid any nonspecific interactions between DNA-coated colloids and glass surfaces of the imaging chambers, coverslips 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 10 µl of each population of DNA-coated colloids, reaching a
final particle volume fraction of $\phi \approx 0.004$, the chamber is completely sealed with glue.

### A.5.5 Confocal imaging

All suspensions were imaged by means of 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 colloids coordinates and to reconstruct 3D images [2].

### A.6 Acknowledgements

I would like to thank Dr. J.H. van Maarseveen and Dr. J.W. Back (University of Amsterdam) for their help with creating a protocol to covalently bind DNA to colloids and Dr. W.T. Kok and L. van Buuren (University of Amsterdam) for determining the yield of these reactions with liquid chromatography.