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Direct observation of size fractionation during colloidal crystallization

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Abstract
We present a confocal microscopy study of the quasi-two-dimensional crystallization of a binary mixture of spherical colloids coated with long DNA strands. Our experiments show that in the crystalline phase the two colloidal species are completely demixed. Analysis of the lattice spacings in the two types of colloidal crystal shows that the diameters of the two species of colloids differ by 10%. We argue that the demixing in the crystalline phase is due to size segregation during crystallization. This phenomenon had been predicted in several theoretical studies. To our knowledge, the present study provides the first 'real-space' experimental confirmation of this effect.

1. Introduction

Crystallization in two- and three-dimensional hard-sphere systems has been studied extensively by theorists and experimentalists since the early 1950s when Alder and Wainwright and Wood and Jacobson performed simulations that showed first-order freezing in a system of hard spheres [1, 2]. Subsequently, Alder and Wainwright studied the melting transition in a system of two-dimensional hard disks [3]. The first numerical estimates of the coexistence points were reported by Hoover and Ree [4]. The study of hard-sphere and hard-disk models received added relevance when, in the 1980s, colloidal systems were prepared that behave effectively as hard spheres (see e.g. Pusey and van Megen [5]). However, the experimental systems exhibit a feature that was absent from the early simulations, namely size polydispersity. In fact, as is discussed in some detail in [6], there appears to be a maximum polydispersity beyond which hard-sphere suspensions do not freeze on experimental timescales. This issue was addressed in numerical simulations by Kofke and Bolhuis who showed that in a 3D system polydispersity widens the density gap between coexisting solid and liquid phases. Moreover, Kofke and Bolhuis [7] found that the (substitutionally disordered, face-centered cubic) crystal phase of hard spheres cannot be stable at a polydispersity above 5.7%. Subsequently, Fasolo and Sollich [8] presented a theoretical analysis of crystallization in highly polydisperse hard-sphere liquids. These authors concluded that a highly polydisperse liquid would crystallize into two or more distinct crystal phases that would contain particles from different 'slices' of the particle-size distribution such that particles with sizes that differ by more than approximately 6% would not be incorporated into a single crystal. In the special case of binary mixtures of spheres with different sizes, this effect had been predicted by Barrat and Hansen [9] and by McRae and Haymet [10]. Computer simulations by Kranendonk and Frenkel [11] for hard-sphere systems in 3D and density functional theory for hard disks in 2D by Xu and Baus [12] did indeed find evidence for this effect. Furthermore, the freezing of quasi-two-dimensional systems of hard colloids was studied numerically by Pronk and Frenkel who used the approach of [7] to show that 2D crystals can support a higher degree of polydispersity than 3D crystals [13]. In particular, they found that stable 2D hard-disk crystals can exist with polydispersities up to 10%. Beyond that degree of polydispersity, we should expect to observe the formation of different, size-fractionated crystals.

In spite of the sustained interest in the freezing of polydisperse colloidal systems, the direct experimental observation of size fractionation upon crystallization into multiple crystal phases has proven difficult. In the present paper we use confocal microscopy to present direct experimental evidence that bi-disperse colloidal solutions with a size ratio of 0.9 fractionate upon crystallization to form

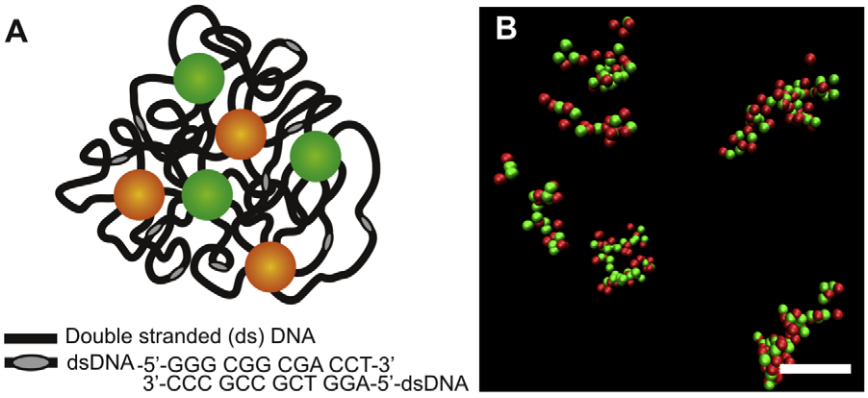

Figure 1. (A) Schematic illustration of red and green fluorescent colloids, grafted with double-stranded DNA (e.g. λ- or pBelo-DNA), carrying complementary single-stranded ends cos 1 and cos 2 that can bind reversibly. Hence, red can only bind to green colloids but not to red, and vice versa. (B) Reconstruction from a stack of confocal microscope images of λ-DNA-coated 1 μm large colloids after an aggregation time of one month. Finite size clusters are clearly visible. The scale bar corresponds to 10 μm.

effectively pure two-dimensional crystals of the larger and of the smaller particles. The system that we study consists of colloids coated with long polymers. Hence, the particles do not behave as purely hard spheres. However, as the stacking in the crystal phase is still determined by the colloidal hard core radius, the physical origin of fractionation upon crystallization should be the same as in the case of pure hard spheres. Moreover, though the resulting two-dimensional crystals are bound irreversibly, their kinetics of formation appears to be consistent with quasi-equilibrium 'reaction-limited' conditions. This is surprising, as the kinetics of sorting can have a strong influence on the resulting crystal morphology [14].

2. Experimental system

In an earlier publication [15] we reported the observation of aggregation of mixtures of red and green fluorescent polystyrene colloids, grafted with a monolayer of long double-stranded λ-phage DNA in solution. The free ends of the grafted DNA are terminated by single-stranded (ss)DNA overhangs such that the red fluorescent colloids with ssDNA cos 1 can only bind to green fluorescent colloids that carry the complementary ssDNA cos 2 (figure 1(A)). At room temperature, in Tris-HCl solutions at pH 8, the binding energy of the cos 1/cos 2 pair is about -15 kcal mol^{-1}, which corresponds to $-25k_BT$. Binding between equally colored colloids is forbidden. The resulting three-dimensional aggregates are finite-sized and disordered (figure 1(B)). Furthermore, they are well mixed meaning all clusters contain roughly equal amounts of red and green colloids. The finite size is a result of the fact that the radius of gyration of λ-DNA, $R_g \approx 800$ nm, is similar to the diameter of our colloids, which is about 1 μm. Consequently all colloids are grafted with about 10λ-DNA polymers [15]. The energy needed for the polymer to reach around the colloid is in the order of k_BT. Hence, in principle all sticky overhangs of a red colloid can bind with those of a neighboring green one. Obviously, there will be a distribution of the number of DNA arms per colloid and a red colloid can simultaneously bind to more than one green colloid. Therefore, the resulting finite size clusters have a larger colloid number density distribution, but usually do not grow indefinitely. We have shown, however, that percolating clusters can be obtained, when the polymeric DNA spacer is shortened: pBeloBac11 DNA (referred to later as pBelo-DNA) can be opened with a restriction enzyme (λ-terminase) such that we have the same sticky overhangs as λ-DNA, but a shorter length dsDNA (7500 bp). With this type of DNA a grafting density of approximately 25 DNA arms per colloid is reached [16]. The radius of gyration of pBelo-DNA is only ∼200 nm, which makes it impossible for the polymer to stretch sufficiently. Consequently, all growing clusters eventually bond together forming a system-spanning fractal network. Interestingly, none of these 3D systems formed crystalline clusters.

However, in a recent publication [17] we showed that, following a different approach, it is possible to grow large, 'floating' two-dimensional crystals of colloids coated with long DNA. In this case, specific DNA binding plays no role. Rather, we graft blunt pBelo-DNA (no sticky overhangs) to one type of colloid, say the red fluorescent ones, and expose a very dilute suspension (volume fraction $\sim 4 \times 10^{-4}$) of them to a surface grafted with a monolayer of polylysine–polyethylene glycol (PLL–PEG; figure 2(A)). The quasi-2D crystals (figure 2(B)) obtained in this scenario are not anchored to the surface through hybridization (although this is also possible) but mainly through the Coulomb attraction between the positively charged polylysine on the surface and the negatively charged DNA bound to the colloids (no free DNA is present in solution). We proposed a two step mechanism that can account for the observed 2D aggregation. In an initial step the colloids diffuse through the density matched sample until the negatively charged DNA corona comes close enough to the PLL–PEG layer to feel a net attraction. As the DNA arms have a gyration radius of almost 1 μm these weakly anchored colloids have enough freedom to still diffuse locally above the surface. This anchoring gives neighboring colloids the time to overcome the weak steric polymer layer around the

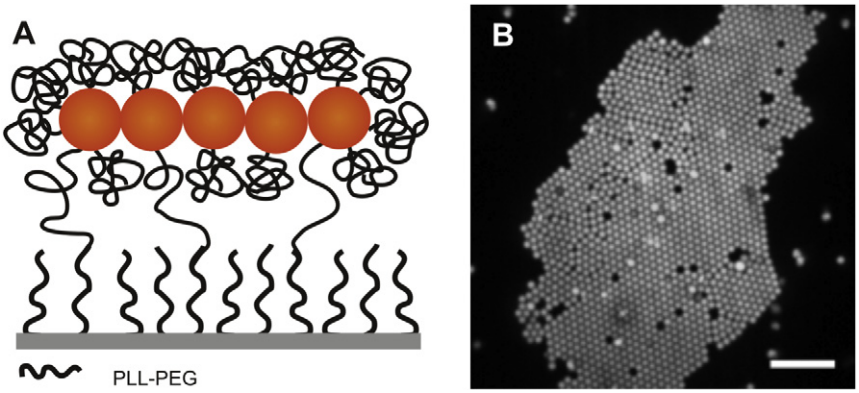

Figure 2. (A) Illustration of 2D crystallization of 1 μm sized colloids coated with pBelo-DNAs that have no sticky cos-overhang. Thus colloids cannot bind to each other while the DNA is weakly attracted to the PLL–PEG surface layer. Note the illustration is not to scale. (B) Confocal image of red fluorescent colloids that have crystallized into a 2D carpet floating about 2.5 μm above the PLL–PEG surface layer. The crystal formed within 2 h, starting from a colloidal volume fraction of $\sim 4 \times 10^{-4}$. The image is a height-projection of several confocal layers (taken over 5 μm) to visualize the entire 2D carpet. Scale bar measures 10 μm.)

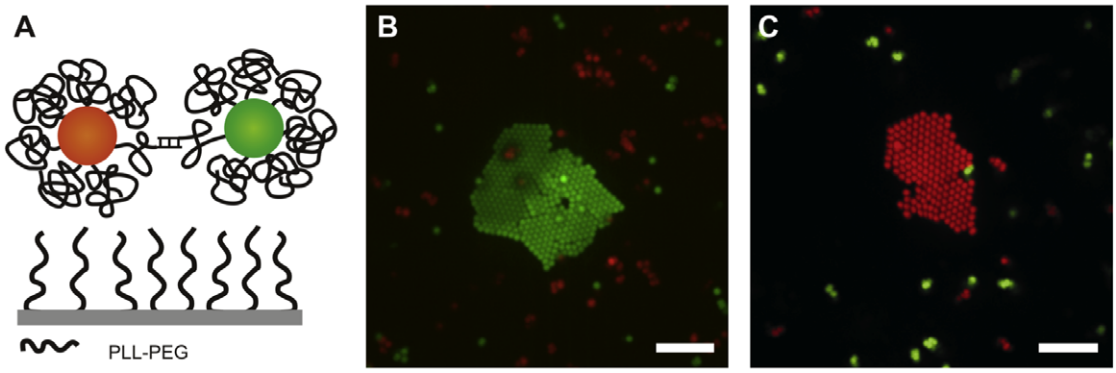

Figure 3. Colloidal 2D crystallization close to a PLL–PEG-coated glass surface from a density matched sucrose-buffer solution containing equal amounts of red and green colloids. The colloids are coated with λ-DNA, carrying cos 1 and cos 2 overhangs. Red and green colloid solutions were mixed seconds before entering the sample chamber. Hence, initially all colloids are dispersed. (A) Schematic representation of the system, indicating that red–green binding is allowed. Confocal images taken in the same sample but different places show that separately red (B) and green (C) colloidal carpets are formed after 3 h. Both images are height-projections of several confocal layers (taken over 3 and 2.5 μm, respectively) to visualize the entire 2D carpet. The scale bars correspond to 10 μm.

colloids. Once this barrier is overcome the colloids fall in a strongly attractive secondary van der Waals minimum[4]. The depth of this minimum will be altered by the grafted DNA monolayer and thus remains difficult to estimate. Although these 2D aggregates will not melt, they do resemble near-equilibrium structures showing the characteristic facets of crystals. Interestingly, no 2D crystallization was observed when using colloids grafted with a cos-overhang. Thus the length of the polymeric spacer and their grafting density on the colloids play an important role as well [17].

In the present work we study the surface-induced aggregation of the same mixtures of red and green fluorescent colloids that we studied previously in bulk solution. In this case we expect to observe weak adsorption of the λ-DNA-coated colloids to the surface but we should also expect to observe binding between red and green colloids due to hybridization of the complementary single-stranded overhangs that terminate the ends of the DNA arms (figure 3(A)). As these systems form mixed, disordered clusters in the bulk we expected to observe, in the presence of positively charged surfaces, the formation of mixed two-dimensional aggregates, possibly crystalline. Figures 3(B) and (C) clearly show that this is not the case. Rather, separate carpets of either only red or only green fluorescent colloids form. Moreover, both types of carpets have similar 'cruising' heights. This observation is surprising as there seemed to be no *a priori* reason why colloids that differ only in their sticky ssDNA ends and in their fluorescent label, would demix on the surface and not form random clusters in the bulk. To gain insight in the physical origins of the demixing, we measured the radial distribution function $g(r)$ of both types of crystals. Interestingly, this analysis revealed that $g(r)$ has a first correlation peak at 1.01 μm $\pm$ 0.02 μm for

[4] Using a standard expression for estimating the van der Waals interactions between two identical spheres, and a Hamaker constant for polystyrene in pure water $A = 9.5 \times 10^{-21}$ J K^{-1}, the attractive interactions are about $300 k_B T$, when the spheres are almost touching. Here we use sugar water, which will have an even smaller Hamaker constant. Zeta-potential measurements give us about 20 mV for the bare neutravidin-coated PS-beads in Tris-HCL buffer at pH 8, corresponding to a repulsive interaction of about $80 k_B T$ at a separation of 1 nm. The resulting DLVO interactions suggest a secondary minimum.

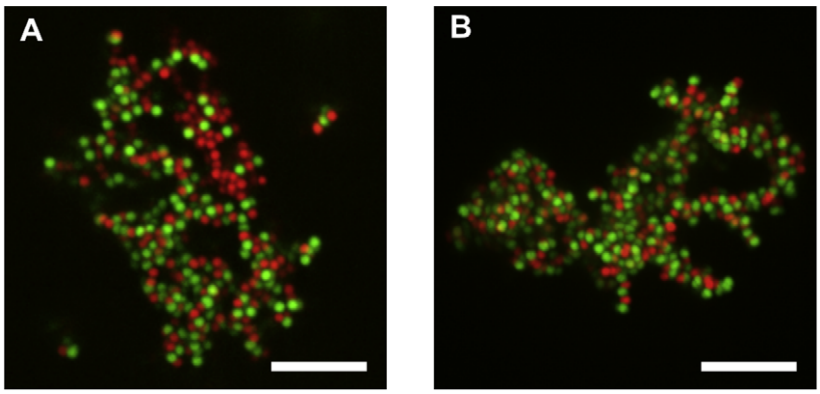

Figure 4. Overlay of several confocal images to show the 3D clusters formed in solution by (A) pBelo-coated PS-colloids with the same sticky overhangs as λ-DNA, and (B) red and green fluorescent PS-colloids coated directly with the single-stranded overhangs cos 1 and cos 2, respectively. Both experiments were done in sample chambers whose glass bottom was coated with PLL–PEG. The cos-coated colloids aggregate within a 30 min after mixing, while the pBelo-clusters form after a few hours. The scale bars correspond to 10 μm.

the red and at 0.90 μm $\pm$ 0.08 μm for the green crystals[5]. As the 2D crystals are effectively close packed, this difference in $g(r)$ indicates that the red and green colloids have a different diameter—even though nominally (that is, according to the specifications) the red and green colloids have the same size. Since the red and green colloids differ by about 10% in size, we are in the regime where size fractionation can take place during crystallization, as opposed to bimodal systems of colloids A and B with larger size ratios for which mixed AB_x (x being a positive integer) crystals can form [18]. The timescale for this two-dimensional crystallization is much faster than the one associated with the hybridization of complementary ssDNA overhangs under the present experimental conditions. Hence there is indeed sufficient time for size segregation to occur. To investigate the competition between size segregation and DNA-mediated binding, we repeated the experiments but with the λ-DNA arms replaced by the (shorter) pBelo-DNA. From our earlier bulk measurements we know that colloids coated with pBelo-DNA hybridize much faster than those coated with λ-DNA. We find that colloids coated with pBelo-DNA do not form 2D crystals but mixed fractal clusters that resemble the corresponding bulk structures. Figure 4(B) shows such a random cluster. Similarly, colloids grafted only with the very short cos 1 and cos 2 oligonucleotides form only disordered clusters (figure 4(B)). This is not surprising as such DNA-coated colloids bind on a timescale that is much shorter than the timescale for 2D carpets to form. In both cases the clusters contain similar amounts of red and green colloids.

By comparing the relative aggregation speeds of the different types of DNA-coated colloids, we can arrive at a qualitative explanation for the observed competition between bulk aggregation and surface adsorption. The colloids coated only with cos-oligonucleotides bind on contact by DNA hybridization. Aggregation takes place in minutes, i.e. in a time that is much shorter than the time needed to adsorb onto the substrate. Colloids coated with pBelo-DNA can in principle form carpets but they can also aggregate in the bulk. Which of the two scenarios dominates depends on the relative rate of the two processes. In the present experiments, only three-dimensional structures were found. This is not surprising because, under the conditions of these experiments, the formation of carpets with colloids coated with pBelo-DNA takes 5–7 h. However, appreciable bulk clustering of pBelo-coated colloids can take place on this timescale. In fact, we know from these earlier measurements that in 5–7 h pBelo-clusters can form that contain up to ∼90 colloids. The clustering via DNA hybridization is therefore faster than carpet formation. Because of the lower concentration of λ-DNA per colloid and its larger extension their aggregation speed is slower than that of pBelo-DNA-coated colloids. This explains why we see carpet formation in the λ-DNA-coated colloids and not with the other two types of DNA-coated colloids.

Finally, we emphasize the hexagonal symmetry in our 2D crystals. In general, we observe that these hexagonal carpets rarely appear as single crystals but contain typical crystal defects such as defect lines, and point defects. However, the question arises whether we have true hexagonal symmetry or rather a hexatic phase. Already in 1966 Mermin and Wagner gave theoretical prove that true long-range translational order is not possible in a two-dimensional crystal [19]. However, on the length scales of our crystals the algebraic divergence of fluctuations is too small to be observed and we can also not distinguish between long-ranged and algebraic bond order.

3. Conclusion

To summarize, we have shown that colloids grafted with very long polymers like DNA can exhibit a new type of 2D aggregation behavior close to a weakly attractive surface. This aggregation behavior is ruled by the strength of the steric stabilization and their aggregation dynamics. Both are a direct consequence of the extreme length of DNA that cannot be achieved for synthetic polymers. It is this polymer-mediated 2D crystallization near an attractive surface that also allowed us to observe phase segregation into two crystals of slightly different lattice spacing due to polydispersity.

[5] Radial distribution functions were obtained by averaging over 6 different carpets obtained from two independent sets of experiments performed in Amsterdam and in Cambridge. For this particular system we obtain reproducibly size-segregated 2D crystals.

4. Materials and methods

4.1. Preparation of the glass surfaces

We used 96 well-plates (Sensoplate; Greiner bio-one) with a glass bottom as sample chambers. The wells were first rinsed with a strong soap solution (Hellmanex; 10% solution) for at least 5 h. After removing all the soap and rinsing the wells with double-distilled (dd) water we added a polylysine–poly(ethylene glycol) polymer solution (PLL–PEG (0.5 mg ml^{-1}; 50 μl); surface solutions). Subsequently excess of unbound polymer was removed by thorough rinsing with dd-water.

4.2. Preparation of biotin–DNA

Two different double-stranded DNA polymer lengths were used. The longest one was λ-phage DNA, which is predominately circular at room temperature. It was linearized by heating 25 μg ml^{-1} solutions to 65 °C, rendering the double-stranded (ds)DNA polymers with two complementary 12 base single-stranded (ss) overhangs denoted cos 1 and cos 2. The linearized DNA was mixed with a solution of short single strands of 12 bases (5′-AGGTCGCCGCCC-3′) with a biotin attached to the 3′-end (5 μl; 20 μM; Eurogentec). To hybridize the oligonucleotides to the DNA, the solution was heated to 65 °C for ∼30 min 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.

The pBeloBac11 plasmid was purchased as a strain (New England Biolabs, ER2420S). To obtain sufficient DNA for our experiments we used bacterial yeast cultures as described elsewhere [15]. The plasmid contains the same 12 basepair-site as λ-phage DNA and can be opened by restriction with λ-terminase (BIOzymTC). The pBeloBac11 DNA was then modified with biotin according to the same protocol as described above.

4.3. Preparing DNA-coated colloids

To obtain DNA-coated colloids the biotin–DNA was mixed with neutravidin-coated red fluorescent polystyrene colloids (diameter 1 μm, molecular probes; exact size see main text) dispersed in a mixture of Tris-HCl buffer (250 mM, pH = 8). In each case the DNA and colloids were reacted overnight during which they were continuously tumbled. Subsequently, the 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 and washed once in a NaOH solution (0.15 μM) to remove poorly bound DNA. The DNA-coated colloids were then diluted in a fresh mixture of Tris-HCl buffer (100 mM final concentration, pH = 8) to obtain a 0.5% solution. For a typical experiment 15 μl of the colloidal solution was added in 200 μl sucrose-Tris buffer (150 mg ml^{-1} sucrose; 100 mM Tris). Similarly green fluorescent colloids (diameter 1 μm, molecular probes) were grafted such that the free end carried the complementary cos-overhang.

4.4. Mixed samples and confocal imaging

Aliquots of DNA-functionalized red and green fluorescent colloidal solutions were mixed seconds before being introduced into the well-plate sample cells (typical colloidal volume fractions used in all experiments were $\sim 4 \times 10^{-4}$). Hence, initially no clusters were present. All suspensions were imaged by means of an inverted microscope (DMIRB, Leica) with a confocal spinning disc 90 scan head (CSU22, Yokogawa Electric Corp.) and a 60× water immersion objective. Fluorescence of the two populations of particles was excited at 488 and 512 nm. Emission was observed at 505 nm and above 600 nm, respectively.

All experiments were repeated several times to ensure reproducibility.

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