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Self-Assembly, Pre-Assembly, and Configurational Reactions of Shape-Designed Colloids in Fluctuating Brownian Monolayers

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Publication Date
2017

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Self-Assembly, Pre-Assembly, and Configurational Reactions of Shape-Designed Colloids in Fluctuating Brownian Monolayers

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Materials Science and Engineering

by

Po-Yuan Wang

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ABSTRACT OF THE DISSERTATION

Self-Assembly, Pre-Assembly, and Configurational Reactions of Shape-Designed Colloids in Fluctuating Brownian Monolayers

by

Po-Yuan Wang

Doctor of Philosophy in Materials Science and Engineering

University of California, Los Angeles, 2017

Professor Thomas G. Mason, Co-Chair

Professor Bruce S. Dunn, Co-Chair

Self-assembly is a promising method for creating new materials. When building blocks are slowly crowded to induce self-assembly in the presence of Brownian excitations, their shapes play important roles in determining the densities at which these building blocks jam and the type in the resulting structures. In this thesis, we describe how to design and lithographically fabricate microscale annular sector particles (ASPs) and disperse them in a solution containing surfactant and depletion agent to form a stable two-dimensional (2D) system. After slowly osmotically compressing these ASPs in 2D using a gravitational force, we observe that a high percentage of
lock-and-key dimers exists in the high density region. We determine the area fraction of these ASPs, thereby obtaining the system’s 2D osmotic equation of state and the dimerization equilibrium constant $K$. We find that $K$ increases exponentially with this 2D osmotic pressure.

Beyond achiral C-shapes, we have been inspired by dimer crystals of chiral proteins, so we have created systems of lithographically fabricated proteomimetic colloids to explore how monolayers of Brownian colloids self-assemble during slow crowding. We modify these ASPs by adding a circular head to only one terminal edge and force the dimerization pathway to select only one chirality through steric suppression. Time-lapse video microscopy reveals that enantiopure dimers crystallize and grow through a kinetic mechanism that we have identified and called tautomerization translocation reactions, which lead to the expulsion of monomer defects from crystallites. We also show that the types and structures of dimer crystals can be tuned by shaping or "lithographically mutating" the location and size of the head on the chiral monomers.

Self-assembling multi-scale hierarchical superstructures that have few defects through slow crowding is an extremely difficult challenge. To overcome this, we utilize photolithography to create pre-assembled complex superstructures that are defect-free. Moreover, we demonstrate that 2D liquid quasi-crystals can be created by the combination of photolithography and chemical release processes. We have precisely pre-assembled Penrose kite-and-dart tiles at high density into a pentagonal quasi-crystalline pattern on the designed mask, and release these particles into a specially optimized solution-dispersion while maintaining the monolayer. We use high resolution time-lapse optical microscopy to study the equilibrium dynamic behavior of fluctuating Brownian kite-and-dart tiles, motifs, and superstructures long after they have been released from the substrate. Moreover, by removing a confining wall, we reveal the melting of this fluctuating quasi-crystal through a pentatic liquid quasi-crystalline regime.
The dissertation of Po-Yuan Wang is approved.

Yu Huang

Jaime Marian

Thomas G. Mason, Committee Co-Chair

Bruce S. Dunn, Committee Co-Chair

University of California, Los Angeles

2017
To my family, friends, and people who have supported me.
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ACKNOWLEDGEMENTS

This dissertation cannot be completed without a full support from the people around me. At this final stage of pursuing a Ph.D. at UCLA, I would like to express my sincere appreciation to everyone who has offered support, inspiration, and encouragement throughout this journey.

First and foremost, I would like to thank my Ph.D. advisor, Prof. Thomas G. Mason, who offered me this huge honor to join his distinguished research group. He not only guided me to solve the practical research problems but also showed me how to be a successful scientist. Through sharing his academic experience, continual insights, and encouragement, Prof. Mason helped me conquer the challenges from both research and life. Without his guidance and persistent support, this dissertation would not have been possible. I am proud to be a graduate student in his lab.

I would also like to thank Prof. Bruce Dunn for taking me as his graduate student in the Department of Materials Science and Engineering. He gave me the directions for preparing graduate study in my first year at UCLA. Appreciation is extended to both Prof. Yu Huang and Prof. Jaime Marian for serving on my thesis committee. Thanks for the insightful and useful suggestions to my research and thesis.

I am glad to work and hang out with the intelligent and fun people in Tom’s group. As I began to work on these projects, I really thank Dr. Kenny Mayoral and Dr. Laura Rossi for teaching me photolithography techniques and knowledge. We spent lots of time solving fabrication problems in the cleanroom at CNSI. I thank Prof. Manas Khan for the valuable discussions for the experimental setup and the simulation calculations. I also treasured all the opportunities to have discussions and fun times with all other members in my group: Prof. Kun
Zhao, Dr. Xiaoming Zhu, Mr. Dimitri Bikos, Mr. Ha Seong Kim, Mr. Matthew Pagenkopp, and Mr. Orin Yue. I will remember the moment that we worked together and laughed together. Special appreciation is also extended to ISNC staff: Ms. Lorna Tokunaga, Mr. Tony Wright, and Ms. Krissy Do. Thanks for all of your help in the cleanroom.

I appreciate the friendships I formed here with Dr. Chun-Han (Matt) Lai, Dr. Charlotte Chou, Dr. Chi-Ping Liu, Dr. Yuan-Wei Chang, Ms. Jean Chiang, Ms. Chia-Jung Yu, Dr. Wei-Hsuan Chang, Ms. Ya-Ting Yeh, and Mr. Yao-Tsung Hsieh. I enjoyed every moment with you. Finally, Matt and Charlotte, we did it! Also, special thanks to those my friends around the U.S., Dr. Tzung-Han Lai and Mr. Shih-Change Hung, you guys encouraged me a lot during this journey. All of you make my graduate memory more precious.

Last but not least, I would like to thank my parents, my sisters and all other family members for supporting me to pursue my Ph.D. degree without any hesitation. My deepest expression of appreciation goes to my wife, Ya-Hsuan Chuang, and my little one, Chloe Wang. Thank you, Ya-Hsuan, for all your love, support, and encouragement, I love you! To my sweet Chloe, even you always distracted me when I was writing thesis. It still was a great time to write with you. We both love you, Chloe. Without the love and support from these mentioned people, my dreams for completing a doctoral degree would have never come true.

submitted. Portions of the herein thesis have been copyrighted previously by the authors in these listed works and are used with permission. Anyone wishing to reproduce any portion of the herein dissertation that is also found in one of these works should contact the respective journals and authors to obtain appropriate permission. Symbols assigned to certain variables in Chapters 2, 3, and 4 may differ according to the specific chapter in which they appear.
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Chapter 1 – Introduction

Colloidal assembly has attracted much interest over the last two decades. Not only does it provide a route for forming colloidal crystals, but it also can be used to mimic and to study phase transitions, providing a view of collective spatio-temporal dynamics that can often be accessed using optical microscopy. Colloidal materials typically have a continuous phase, which is usually a liquid or fluid (dispersion medium) and a dispersed phase, which is usually made up of many microscale to nanoscale objects (often composed of solids or immiscible liquids) that are subject to Brownian fluctuations. Based on the phase of dispersed substance and dispersion medium, colloids can be classified as follows: a sol (solid particles dispersed in a continuous liquid medium); an emulsion (liquid droplets dispersed in an immiscible liquid medium); a foam (gas bubbles in a liquid or solid medium); or an aerosol (solid particles or liquid droplets dispersed in a gas)\(^1\). Colloidal particles, ranging from a few nanometers to about 10 micrometers, are often synthesized by a bottom-up chemical synthetic approach; in some cases, the particle size and shape can be controlled with exquisite precision. These highly uniform particles can self-assemble into colloidal crystals or hierarchically organized structures with the assistance of external forces\(^2\)-\(^6\). The kinetic mechanisms of colloidal crystallization are still not fully understood at this stage, especially the relationship between the shapes of particles and the final crystal structure. In this thesis, we focus on 2D bottom-up self-assembly and top-down pre-assembly of aqueous dispersions of microscopic solid particles having different customized shapes made using top-down lithography. Top-down fabrication provides access to certain colloidal shapes that are currently unavailable through bottom-up synthesis routes.
In a colloidal dispersion at a temperature $T$, the dispersed particles and molecules in the liquid dispersion medium are subject to entropic fluctuations related to the second law of thermodynamics. Exploration of accessible microstates, associated with maximization of Boltzmann's entropy, leads to the random fluctuating motion of the colloidal particles in the liquid even while it is in equilibrium. This random fluctuating motion of colloidal particles was first observed microscopically by Robert Brown in 1827. In 1905, Albert Einstein provided a theory for Brownian motion which predicted that the diffusion coefficient is proportional to $T$ and inversely proportional to the size of the particles and to the viscosity of the continuous liquid. In 1909, Jean Perrin experimentally verified Einstein’s equation for Brownian motion and famously estimated Avogadro’s number $N_A$.

In addition to Brownian motion, repulsive forces and attractive forces between particles play important roles in colloidal stability. The repulsive forces, including electrostatic and steric interactions, inhibit colloidal particles from aggregating and flocculating. Electrostatic repulsions depend on the distribution of charges over the particles; these repulsions can typically be tuned by adding salt or adjusting pH of the solvent to change the ion concentrations in the continuous phase. Attractive van der Waal forces result from differences in charge fluctuations between atoms or molecules of different types. The combination of these forces into a single interaction potential can explain the conditions of colloidal stability in some standard colloidal systems and forms the basis of the Derjaguin-Landau-Vervey-Overbeek (DLVO) theory. For instance, surfaces of particles can be stabilized using adsorbed dodecyl sulfate surfactant anions (DS-). Due to the absorbed DS- on their surfaces, the particles repel each other strongly at short distances through screened charge repulsions having a Debye length of a few nanometers. For
microscale particles that are much larger than the Debye screening length, such short-range repulsions yield effectively hard interactions.

1.1 Colloidal Assemblies in Monolayers

Because colloidal particles can have structural similarities with atoms and molecules as well as being large enough to be easily imaged under high-resolution microscopy, colloids can sometimes serve as nice model systems for studying fundamental non-quantum behavior of condensed matter. For instance, intensive research has been conducted to understand the microscopic kinetics of crystallization\(^\text{12}\), melting\(^\text{13}\), defect formation\(^\text{14}\), and glass transitions\(^\text{15-16}\) using colloidal model systems. Furthermore, spontaneously formed periodic structures of monodisperse colloids that have highly uniform size distributions provide routes for fabricating photonic crystals that have important applications in photonics, biosensors, and optical communications\(^\text{17-18}\). Colloidal particles can form one-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) structures based on the dimensionality of the assembly. In a 1D structure, the colloidal assembly only extends in one direction such as chain of particles. In a 2D structure, the colloidal particles form monolayer arrays in two-dimensional directions such as graphene. On the other hand, a 3D structure has multilayers of ordered arrays of colloidal particles such as photonic crystal. Several techniques have recently been created and developed for precisely positioning colloidal particles in 1D\(^\text{19}\), 2D\(^\text{20}\), and 3D\(^\text{21}\) structures. Herein, we focus on new methods of self-assembly and pre-assembly of 2D monolayers of plate-like microscale colloidal particles, or "tiles", that we fabricate using top-down lithographic methods.

Colloidal monolayers can be used not only as templates for fabrication of patterned nanostructures but also as readily observable dynamic systems for studying phase transitions.
Several assembly strategies for forming low-defect and high-ordered colloidal monolayers have been experimentally demonstrated previously\textsuperscript{22-23}. Conceptually, all these assembly techniques for close-packed monolayers can be classified in two categories: liquid interface-mediated assembly and direct assembly on or just above a solid substrate. In liquid interface-mediated methods, the colloidal particles crystallize at the interface between two liquids or on the surface of a liquid. Colloidal crystallization at a liquid interface is mediated by capillary interactions linked to interfacial tension. By changing interfacial and wetting energies, particles can be trapped at the interfaces and assemble into different structures\textsuperscript{24}. In direct assembly methods, a colloidal crystal can self-assemble through evaporation-induced processes or through processes involving external forces. For evaporation-induced methods, colloidal particles can freely diffuse over a substrate and can form the lowest energy configuration associated with capillary forces when the solvent evaporates. On the other hand, using external forces including gravity, shear forces, electrostatic forces, and electrophoretic forces, the colloidal particles can be guided to crystallize and form different structures. For colloidal systems, the role of random entropic fluctuations can also be significant, and the degree to which random Brownian forces might dominate over such externally applied forces is likely to be important in determining resulting structures (e.g. as quantified by a Peclet number for shear forces).

\subsection*{1.1.1 Assembly Interactions – Depletion Attractions}

Model systems of colloidal hard spheres have been developed and widely studied\textsuperscript{16, 25-26}. Both experiments and simulations demonstrate that this model system can successfully be used for studying crystallization and melting behavior. In the ideal hard sphere model, each colloidal particle is an impenetrable sphere and cannot overlap in space. In terms of energy, the interaction potential of two spheres is infinitely repulsive if they overlap, whereas the potential is zero
otherwise. Since no interaction potential exists between the spheres, the system is governed completely by entropy. The phase behavior of a monodisperse colloidal hard sphere system is connected to its particle volume fraction, $\phi$. For gradual quasi-static osmotic compression beyond $\phi > 0.495$, some spheres can form crystallites spontaneously\textsuperscript{25}. For $0.495 < \phi < 0.545$, the system exhibits a two-phase co-existence between a disordered liquid phase and a crystalline solid phase, and the system becomes completely crystalline at higher $\phi > 0.545$\textsuperscript{25} up to full face-centered cubic (FCC) packing at $\phi_{\text{FCC}} = 0.74$. These phase transitions were found in simulations by Hoover and Ree\textsuperscript{27} and confirmed experimentally by Pusey and van Megen\textsuperscript{25}, who investigated dispersed systems of sterically stabilized polymethyl methacrylate colloids after slow sedimentation. Crystallization in dense colloidal hard sphere systems takes place as a direct consequence of entropy maximization. Thus, this entropy-driven phase transition serves as an important prototype for understanding entropy-dominated forms of colloidal self-assembly.

In addition, entropy maximization can generate an attractive force in binary colloidal dispersions of two types of colloids that have significantly different sizes; the larger colloids experience a depletion attraction that is driven by the smaller colloids. The attractive depletion force between two larger spherical colloids, driven by smaller spherical colloids, assuming hard interactions among all colloids, was first predicted theoretically by Asakura and Oosawa\textsuperscript{28}. In a mixture of large and small colloids, the depletion force arises between large colloidal particles because smaller particles, which are often called depletion agents (or in some cases depletants), are preferentially excluded from the volume between proximate surfaces of two large particles. To understand the depletion interaction between larger colloids and depletion agents, the hard sphere model's canonical ensemble has been used to calculate the free energy of the system and interaction force between large colloids. In this model, the large hard spheres, which have
diameters $D = 2R$ where $R$ is their radius, are suspended in a liquid that also contains small hard spheres, which have diameters $d = 2r$. Around each large sphere, there is a volume that is entropically inaccessible to small spheres due to the colloid-depletion agent hard-sphere potential. This excluded volume is defined as $V_E = 4\pi(R+r)^3/3$. When the large particles come close together, such that their center-to-center distance $h$ is less than $D + d$, then the two excluded volumes surrounding the two large spheres overlap, yielding an overlapping excluded volume $V_{OE}$, as shown in Fig. 1.1. This overlap of excluded volumes effectively increases the volume in the system available for depletion agents to explore (i.e. increases the number of accessible translational microstates for small spheres), and so an attraction between the larger spheres develops as a consequence of the second law of thermodynamics. Said in an equivalent yet different way, when two larger spheres closely approach each other, such that they impede the diffusion of depletion agents in the region between them, the resulting density gradient of depletion agents causes an anisotropic osmotic pressure, leading to an attractive interaction between the large spheres. Strong depletion attractions, relative to thermal energy $k_B T$, can be used to promote flocculation of larger colloids. The strength of the depletion attraction depends on the concentration and size of the depletion agents, and the range of the depletion attraction is typically short and comparable to the size of a depletion agent.

The geometry of a particle also plays a role in the depletion attraction because the overlapping excluded volume is strongly dependent on the surface geometry and morphology of particles\textsuperscript{29-30}. The rough surfaces, which have the size on the order of the depletion agent size, have smaller overlapping excluded volumes than the smooth surface. This provides suppression in depletion attractions as shown in Fig. 1.1. Throughout this dissertation, we employ such
roughness-controlled depletion attractions (RCDAs) to create fluctuating monolayers of micro-sized platelets.

![Diagram of depletion attractions](image)

**Figure 1.1 Schematics of depletion attractions between colloidal spheres and roughness controlled depletion attractions between lithographic platelets and a glass slide.** (a) Two large spherical colloids (yellow circles) having radius, $R$, in a solution of small spherical colloids as depletion agents (green circles) having radius $r$. Around each large sphere, there is an excluded volume (gray) inside which the centers of small depletion agents cannot penetrate. When the distance between two centers of large colloids is smaller than $2R+2r$, the depletion layers of two large spherical colloids begin to overlap (orange region) and form an overlapping excluded volume. (b) Side view of lithographic platelets (blue) near a glass slide. Increased roughness on the surface of colloids reduces the in-plane depletion attractions by decreasing the in-plane overlapping excluded volume (red). The asperities are exaggerated for clarity.
1.1.2 Shapes of Building Blocks for Assemblies

Both simulations and experiments have demonstrated that the shapes of building blocks in a system can influence their self-assembly, phase behavior, jamming, and packing\textsuperscript{31-32} at high densities. At a simple level, to understand colloidal self-assembly and to predict resulting self-assembled structures, different core shapes of particles need to be considered in the context of entropy maximization. Especially as systems of non-spherical colloidal particles begin to become crowded, the shapes of the constituent particles can profoundly affect the system's overall entropy. Nowadays, with advances in chemical synthesis and nanoscale fabrication, particles with different shapes and geometries can be fabricated to yield a high degree of uniformity and sometimes also can be mass-produced in the laboratory. In a 2D system, colloidal particles with convex shapes, such as the circle\textsuperscript{20}, square\textsuperscript{33}, kite\textsuperscript{34}, and rhombus\textsuperscript{35}, have been studied and their self-assembled structures have been identified at different particle area fractions. Beyond convex polygonal particles, concave polygonal particles that have concave features (\textit{i.e.} that have one or more interior angle greater than 180 deg), such as square crosses\textsuperscript{36} and tri-stars\textsuperscript{37}, can have interesting phase behavior based on interdigitation of sub-particle protrusions. Overall, the colloidal particles with concave geometries have not received as much attention as the convex particles. These shapes potentially offer new mechanisms for assembly, such as interpenetrating lock-and-key structures, which are not allowed in systems of convex particles\textsuperscript{38}.

This dissertation is organized as follows. First, we focus on the self-assembly of concave particles and fabricate C-shaped tiles, which we also formally call annular sector particles (ASPs), using photolithography. Two ASPs can interpenetrate and form a lock-and-key chiral dimer. A racemic mixture of such randomly formed chiral dimers leads to dense disordered systems, as we show in Chapter 2. To form a dimer crystal and avoid racemic disorder, we
purposefully add a circular head on only one side of ASP to sterically select only one enantiomeric dimer chirality. Using shape design to modify reaction pathways during slow crowding, we form enantiopure lock-and-key chiral dimers; these, in turn, form chiral dimer crystals, as we show in Chapter 3. This hierarchical self-assembly of dimer crystals is reminiscent of dimer crystallization in proteins and may help explain this for certain types of proteins. However, other multi-scale colloidal structures have not yet been self-assembled because their organizations are much more complex. For instance, the Penrose tilings, composed of two different building blocks, have quasi-crystalline order in contrast to traditional definitions of crystallinity based on translational symmetry. Because of the complex features of Penrose tilings, particularly their 5-fold symmetry, existing particle shapes have difficulty self-assembling into a Penrose quasicrystalline pattern. We, therefore, have developed a new method for fabricating colloidal particles in a highly complex pattern. We pre-assemble and fabricate kite-and-dart particles with a quasicrystalline Penrose pattern using a photolithography processes. After releasing the Penrose tiles from the substrate, yet preventing these released tiles from leaving a monolayer using anisotropic depletion attractions, we study the resulting fluctuating Brownian Penrose quasicrystals, as we present in Chapter 4.

1.1.3 Creating a Monolayer: Self-Assembly by Slow Crowding

To measure and analyze complex spatiotemporal dynamics in dense systems of complex-shaped particles, we first fabricate micro-sized shape-designed platelets using photolithography. These are suspended in a stabilizing solution, which inhibits aggregation, and then washed several times to remove residual release material by gravitational or gentle centrifugal settling, removal of supernatant liquid, and resuspension in clean stabilizing solution. The resulting dispersion of washed lithographic particles is diluted with an aqueous solution containing sodium
dodecyl sulfate (SDS) as a stabilizer against particle aggregation and nano-sized polystyrene spheres as depletion agents; this dilute dispersion of lithographic particles with depletion agent is then loaded into a flat glass microcuvette. The lithographic particles (or platelets) experience short-range electrostatic Debye screened-charge repulsions provided by the adsorption of amphiphilic DS- ions on the particles' surfaces. This repulsion ensures that a lubricating layer of liquid will persist between the platelets and the cuvette's glass wall. The depletion attractions between the faces of the platelets and the lower glass wall of the cuvette are strong compared to thermal energy $k_B T$; these attractions stabilize the 2D monolayer against particles being thermally excited by occasional strong Brownian fluctuations out of the plane. The roughness on the particles' edges is larger than the roughness on the particles' faces; this anisotropy in surface roughness lowers the in-plane depletion attraction between platelets below $k_B T$. Thus, by tuning and optimizing the concentration of SDS and depletion agents, the platelets can freely translate and rotate in a persistent monolayer without inducing any aggregation. The micro-cuvette, containing platelets and the solution, is sealed on a glass slide using UV optical glue and placed on the tilted flat surface to concentrate the platelets slowly (i.e. quasi-statically) over several months. We observe the fluctuating monolayer using brightfield transmission optical microscopy and record movies of spatiotemporal dynamics at different magnifications using long-working distance objective lenses. The schematics of the experimental set-up are shown in Fig 1.2.
1.2 Photolithography

In modern times, photolithography is one of the key processes in the semiconductor industry, which enables the fabrication of incredibly small size integrated circuits that can be used in smart electronic devices. The basic principle of mass production of electronics based on modern photolithography is repeatedly transferring geometric shapes from one or more pre-designed masks to photo-responsive material on the surfaces of silicon wafers. After ultraviolet (UV) light passes through the patterns on a mask, this patterned UV light triggers photochemical reactions of light-sensitive photoresist that has been previously coated onto a silicon wafer. For negative photoresist, the patterned optical exposure typically causes selective spatial crosslinking of the photoresist which then cannot be easily washed away during development. By contrast, the exposed region in a positive photoresist become more soluble in the developer and can be readily washed away.

While protocols for creating consumer electronics can be considerably more complex, we use a simplified photolithography process that involves wafer cleaning, sacrificial layer coating, photoresist coating, soft baking, UV light exposure, post exposure baking, development, and hard baking. However, this technique also can be developed and used to fabricate, on a mass
scale, colloids in a top-down process. Utilizing direct transfer of images from the mask to the photoresist-coated wafer, highly uniform colloids having various shapes and sizes can be readily mass-produced. Contact lithography and complex step-and-repeat projection (stepper) system are two important tools that can be used to perform high-quality photolithography. While contact lithography using a mask aligner can be advantageous in certain situations, usually stepper systems provide higher resolution, longer mask life, and smaller feature sizes.

Following a procedure introduced by Hernandez and Mason, we fabricate ASPs and Penrose tiles using an ASML PAS 5500/200 i-line 5:1 reduction stepper. The dimensions and shapes of ASPs and Penrose tiles are designed using L-edit software, and the patterns are transferred to a chrome-on-quartz mask using electron beam lithography. Initially, a silicon substrate is chemically cleaned to remove any organic, ionic, and metallic impurities on the surface. Subsequently, the cleaned wafer is spin-coated with a sacrificial and a photoresist layer. The sacrificial layer, composed of polydimethylglutarimide, is soluble in a basic solution. The photoresist layer is a light-sensitive material that selectively crosslinks or decomposes under patterned UV lights. We use a negative photoresist (SU-8 2000 series permanent epoxy resists) to fabricate particles because SU-8 has excellent chemical resistance, better imaging characteristics (e.g. is optically transparent in the visible range), and higher aspect ratio features. Since the thicknesses of the sacrificial layer and of the SU-8 layer are determined by the spin coating rates and flow properties of these materials in solution, we adjust the spin coating rates to achieve a useful thickness of the sacrificial layer (∼125 nm) and to set the thickness of the SU-8 layer (∼1-2 μm). After spin coating each layer, the wafer is soft baked to remove the solvent and solidify the uniform film. The coated wafer is then loaded into the stepper and is patterned by exposing the photosensitized wafer to the UV light (i-line, 365 nm) patterned by the mask. This
patterned UV light contains the geometric features of the designed particles on the SU-8 layer, and the exposed SU-8 begins to cross-link. We then bake the exposed wafer, which increases the rate of crosslinking, and develop the patterned photoresist layer using an organic developer solution. Without the post exposure bake, incomplete crosslinking reactions result in only a very faint pattern or no pattern of photoresist after development\(^4\). In the development process, an organic SU-8 developer solution dissolves away the uncrosslinked SU-8, which leaves patterned crosslinked SU-8 in the form of discrete shape-designed particles on the solid sacrificial layer. Finally, we rinse the developed wafer with isopropyl alcohol to remove any residue of uncrosslinked SU-8 and developer solution. The simple schematics of the photolithographic fabrication of colloidal particles are shown in Figure 1.3.
Figure 1.3 Schematics of photolithographic fabrication of shape-designed colloidal particles. A silicon-wafer substrate is coated with a sacrificial layer of a release material (e.g. 120 nm of LOR1A) and then a photoresist layer (e.g. 1 μm of SU-8). UV light passes through the chrome-quartz mask and initiates crosslinking reactions in the exposed SU-8. After post exposure baking and development, the uncrosslinked SU-8 is dissolved away using an organic SU-8 developer solution. The fully formed SU-8 particles have sizes and shapes that are defined by the mask. Subsequently, the sacrificial layer can be dissolved in an aqueous SDS solution containing TMAH. Adapted from Figure 1 in Hernandez and Mason.

1.3 Summary

In order to understand how particle shapes affect the entropy, structure, and dynamics of many-particle systems, we lithographically fabricate different shapes of micro-sized platelets and slowly concentrate them in fluctuating Brownian monolayers. Inspired by protein dimers, we
design a series of annular sector particles \textit{(i.e.} C-shapes) having different curvatures, and we have revealed that these C-shaped particles can interdigitate with each other to form mutual lock-and-key dimers. In Chapter 2, we create a monolayer of ASPs and slowly concentrate them gravitationally in a slightly tilted 2D column. When the system reaches a near-equilibrium state, the ASPs can form a racemic mixture of dimers in the different regions of the column. This self-assembly of ASPs into dimers can be attributed to the 2D applied osmotic pressure. Since the dimers of ASPs are a racemic mixture of positive and negative chiralities, these two different dimer types frustrate crystallization and instead cause the formation of a structure that is similar to a disordered glass. If one could force the dimerization pathway to only select either positive or negative chirality, by steric suppression for instance, then the enantiopure dimer system could have a much greater proclivity to crystallize, reminiscent of chiral protein crystals composed of chiral dimers. In Chapter 3, we modify C-shaped particles by placing a head on only one of the two protruding arms, which sterically suppresses the formation of one dimer chirality, thereby creating an enantiopure chirality of all dimers in the monolayer. These chiral modified C-shaped particles can serve as a simplified model to mimic chiral dimerization of certain proteins. Therefore, we can use these chiral C-shaped particles to examine how different shapes and entropy combine to influence self-assembly in slow crowding conditions similar to what is used in many protein crystallization protocols.

Going beyond self-assembly, we have created a new lithographic-release technique based on pre-assembled colloidal tiles and a customized solution-dispersion that enables us to study the fundamental physical behavior of fluctuating 2D systems of hierarchically superstructured shape-designed colloids. With this method, which we call lithographically pre-assembled monolayers (Litho-PAMs), we can set the initial configuration of tiles in a monolayer in ways that have not
yet been accessed by self-assembly. For instance, guiding a plurality of two different types of particles to self-assemble into hierarchically organized superstructures, such as those in a 5-fold Penrose kite-dart quasi-crystal, is a very challenging prospect. In Chapter 4, we pre-assemble and directly print micro-sized kite-like and dart-like particles with Penrose tilings using a photolithography process and release these particles in a customized solution-dispersion to form a fluctuating Brownian monolayer. From these observations in real space and associated Fourier transforms (FTs), we obtain evidence of a new 2D phase called a fluctuating pentatic liquid quasi-crystals (LQC5). Time-lapse videos of fluctuating Brownian quasi-crystals diffusing into empty space also reveal their melting behavior. By quantitatively analyzing these videos, we determine the melting point of the fluctuating Brownian system of Penrose P2 tiles. Also, we show that certain pentagonal-star kite motifs in the Penrose pattern can collectively rotate and exhibit heterogeneous dynamics, including collective hopping rotations, in equilibrium. In summary, Litho-PAMs opens up new routes for studying new phases of colloidal matter and their melting, bypassing the often slow kinetics of self-assembly and directed assembly.
References


Chapter 2 – Colloidal Lock-and-Key Dimerization
Reactions of Hard Annular Sector Particles
Controlled by Osmotic Pressure


2.1 Introduction

The simplest structure that can be assembled out of identical monomer building-blocks is the dimer, which is composed of two monomers. A wide variety of dimers occur naturally at everyday conditions. Diatomic molecules are dimers made of atomic building-blocks such as oxygen $O_2$ and nitrogen $N_2$, whereas dimers of capsid coat proteins, such as those of simple bromoviruses, can serve as more complex building blocks for other structures. Many different mechanisms can play a role in dimer formation. For instance, electronic and quantum mechanical pair interactions between monomer atoms can lead to diatomic molecule formation through strong attractions associated with covalent bonding. Alternatively, entropically driven anisotropic roughness-controlled depletion attractions (RCDAs) between plate-like colloids, each of which has a rougher face and a smoother face, can be used to form a stable phase of dimers: the smoother faces of two platelets are attractively bound together inside each dimer and the outer surfaces of each dimer are entirely rough, thereby precluding subsequent aggregation. As a different mechanism, hydrogen bonding can cause self-association of two molecules of acetic acid in certain organic solvents, yielding linear and cyclic dimers. As yet a different example, certain localized site-specific attractive and repulsive interactions between two
proximate monomer protein molecules can also lead to dimer formation into highly reproducible dimer protein structures.\textsuperscript{9-14} Often, the core shapes of these molecules have convex protrusions and also concave receptacles, enabling mutually interpenetrating lock-and-key dimer structures to be assembled.

The formation reaction of two monomer building blocks into a dimer is known as a dimerization reaction, and the decomposition reaction of a dimer into two separate monomers is called a dissociation or a de-dimerization reaction. If the two monomers are identical, then the reaction is a homo-dimerization reaction. For systems composed of many monomers, in which attractive interactions between monomers are the mechanism for dimer formation, the proportions of monomers and dimers in equilibrium at a certain temperature $T$ and a pressure $p$ are well known in physical chemistry. The equilibrium reaction between two monomers has a forward dimerization rate constant $k_+$ and also a backward dissociation rate constant $k_-$, where the equilibrium constant $K$ of the reaction is simply $K = k_+/k_-$.\textsuperscript{8} In cases when attractive interactions between monomers drive dimerization, if the pair attraction has an effective bond energy, $U$, (e.g. associated with a minimum in a potential well), then dimers will dominate when $U$ is much larger than thermal energy $k_B T$. However, if the temperature is raised so that $k_B T$ becomes much larger than $U$, then monomers will dominate.\textsuperscript{7} For many attractive monomer-dimer systems, pressure typically only plays a secondary role.

While equilibrium dimerization and dimer dissociation reactions for attractive monomer building blocks are well-known, regardless of whether or not the two building blocks mutually interpenetrate to form lock-and-key structures, much less is known about dimerization of systems of shapes at equilibrium in which pair interactions are effectively hard, so that only geometrical shape of the monomer and entropy at a certain pressure and temperature control the
degree of dimerization. Moreover, the meaning of dimerization must somehow be defined geometrically for such systems of hard shapes, and the requirement of mutual interpenetration of two monomer shapes into a single lock-and-key dimer structure could be used as this definition.

Unanswered questions exist about the range and types of monomer shapes that could lead to significant populations of dimer structures when Brownian systems of monomers are slowly concentrated to high densities. Particularly, in colloidal systems of solid particles dispersed in a weakly viscous liquid, the advent of shape-designed platelets\textsuperscript{15-18} and the creation of two-dimensional (2D) near-equilibrium systems of these shapes over a wide range of particle densities\textsuperscript{19-20} offers the potential for exploring the behavior of hard shapes that could potentially dimerize into interpenetrating lock-and-key structures. For such 2D colloidal systems, one would expect that the equilibrium constant $K$ of the dimerization reaction would depend on the 2D osmotic pressure, $\Pi_{2D}$, since $\Pi_{2D}$ is related to the area fraction of particles, $\phi_A$, through an osmotic equation of state $\Pi_{2D}(\phi_A)$\textsuperscript{21} Even having a single robust experimental demonstration of the behavior of a lock-and-key colloidal dimerization reaction over a wide range of applied $\Pi_{2D}$ in a thermally excited system would represent an important advance in understanding the fundamental behavior of colloidal dimerization of hard objects.

RCDAs have been demonstrated to provide a robust route for forming 2D Brownian systems of lithographic platelets, thereby enabling experimental exploration of the phase behavior of dense systems having a wide variety of shapes over a range of particle area fractions $\phi_A$. RCDAs keep microscale platelets oriented with their faces parallel to a transparent, solid, smooth substrate, yet in-plane interactions remain effectively hard. Many particles in a plane are then concentrated very slowly by tilting the substrate (e.g. a sealed rectangular cuvette) by a
small angle, so that gravitational sedimentation of the platelets effectively applies a two-dimensional osmotic pressure that varies with the distance along the substrate.\textsuperscript{19}

While 2D Brownian systems of hard shapes explored experimentally thus far have been mostly convex polygons, such as triangles,\textsuperscript{22} squares,\textsuperscript{20} pentagons,\textsuperscript{19} rhombs,\textsuperscript{23} parallelograms,\textsuperscript{24} and kites,\textsuperscript{25} some shapes have forms of concavity, including arms. Four-arm square crosses have been shown to form chiral crystallites at high densities,\textsuperscript{26} whereas slender three-arm tri-star particles form large crystallites of an alternating stripe crystal, reminiscent of steric zippers.\textsuperscript{21}

While these shapes have yielded a wide variety of self-organized structures, thus far, topologically identifiable lock-and-key dimerization in dense Brownian systems of colloidal objects that have hard interactions has not been previously explored experimentally.

In order for two copies of a single, hard, monomer shape to have the possibility of forming a lock-and-key dimer, that monomer shape must necessarily possess both a concave portion and also a convex portion. One of the simplest shapes in 2D that satisfies this requirement is the annular sector.\textsuperscript{27} An annular sector has a perimeter given by four segments: an outer arc that has a radius $R_o$ and a filled angle of $2\pi - \psi$, an inner arc that has a radius $R_i$ and the same filled angle $2\pi - \psi$, and two radial line segments of length $\Delta R = R_o - R_i$ connecting the inner arc to the outer arc. Thus, the opening angle that is not filled is $\psi$, and the annular sector's effective thickness is $\Delta R$. Annular sectors represent an interesting class of shapes encompassing structures ranging from nearly rectangular to pie-like to nearly toroidal (i.e. ring-like). For $0 < \psi < 2\pi$, an annular sector necessarily has an accessible concave portion to its perimeter; the convex portions consist of the outer arc, as well as the ends of two arms that are terminated by the radial line segments. If $R_o$ and $R_i$ are both greater than $\Delta R$ and $\psi R_i$ is also at least somewhat larger than
ΔR, then the ends of the arms will have a significantly larger convex curvature than the concave curvature and one of these arms of a first annular sector can potentially insert reversibly into the opening of a second neighboring annular sector, yielding a lock-and-key dimer configuration. Thus, we design open ring-like microscale platelets that meet these requirements and refer to them as annular sector particles (ASPs). In particular, we focus on ASPs having \( \psi \approx \pi/2, R_o \approx 3 \mu m, \) and \( \Delta R \approx 1 \mu m, \) since this partial ring has an opening angle that offers the potential for dimerization. The size of the ASPs, set by \( R_o, \) is small enough that their Brownian motion is significant, yet large enough that they can be easily observed using an optical microscope.

Here, we show that 2D Brownian systems of designer microscale lithographic ASPs, when slowly compressed osmotically in 2D to large enough \( \Pi_{2D}, \) can dimerize at an efficiency nearing 100\%. Moreover, below a predominantly monomer fluid zone, in the tilted cell of ASPs, we demonstrate that there is a near-surface equilibrium reaction zone. In this spatially resolved reaction zone, dimerization and de-dimerization reactions both occur, so we are able to use particle counting statistics to directly determine how the degree of dimerization, given by the equilibrium constant \( K, \) depends on \( \Pi_{2D}. \) In addition, we reveal the presence of two distinguishably different positive and negative chiralities of dimers in racemic proportions, and show that the spatial pair correlation function, based on the center positions of ASPs, contains a prominent dimer peak as well as two other peaks that are effectively related to the dimensions of the two-ASP dimer unit.
2.2 Materials and Methods

2.2.1 Photolithographic Fabrication of Annular Sector Particles

Using top-down photolithography (ASML stepper, PAS 5500/200, 5X, ultraviolet (UV) i-line 365 nm), we fabricate microscale plate-like annular sector particles (ASPs) composed of cross-linked SU-8 polymer photoresist.\textsuperscript{15} Into this stepper, we load a negative quartz-chrome mask (MEBES, electron beam lithography, 50 nm) that has been designed with a rectangular array of annular sector shapes having a designed opening angle of $\psi = 90.0$ deg, an inner radius of 15.4 $\mu$m and an outer radius of 10.2 $\mu$m as shown in Figure 2.1a. We prepare a water-soluble sacrificial layer on 4-inch silicon wafers by spin-coating LOR-1A (Microchem Inc.), yielding a thickness $\approx 120$ nm after baking at 200 $^\circ$C for 120 s and cooling to room temperature. On the layer of LOR 1A, we spin coat SU-8-2001 (Microchem Inc.) photoresist, which is baked at 95$^\circ$C for 90 s, yielding a 1 $\mu$m uniform SU-8 layer. A coated wafer is loaded into the stepper, exposed to patterned ultraviolet (UV) light, post-baked, and developed in order to remove unexposed and uncrosslinked SU-8. After developing, this wafer is rinsed with isopropyl alcohol and dried with a flow of nitrogen gas, leaving prismatic annular sector particles composed of SU-8 attached to the sacrificial layer of LOR-1A. This sacrificial layer is then dissolved in a basic surfactant solution containing 1.8\% w/v tetramethylammonium hydroxide (TMAH, Sigma-Aldrich 25\% in water) and 40 mM sodium dodecyl sulfate (SDS, MP ultrapure), and the particles lift-off into the solution. The hydrophobic tails of SDS molecules adsorb on the surfaces of the SU-8 particles, providing a high enough surface charge to stabilize the particles against aggregation. By successively concentrating the dispersion, removing the supernatant, and diluting it four times in aqueous solutions of deionized water at 5 mM SDS, we remove any residual dissolved LOR 1A
and TMAH, and we also set [SDS] = 5 mM. By processing three wafers and combining the resulting dispersions, we obtain an aqueous dispersion containing ≈ 300 million stabilized ASPs.

2.2.2 Characterization of ASPs

We use scanning electron microscopy (SEM), rather than optical microscopy, to characterize the dimensions and shapes of the ASPs precisely, since optical diffraction could smear out key features. In Figure 2.1b, we show the face-on view of a typical SU-8 ASP, obtained using JEOL, JSM-6700 FE-SEM, 5 kV. By performing SEM on 10 different particles, we determine the average outer radius to be \( R_o = 2.8 \pm 0.1 \) \( \mu \)m and the average inner radius to be \( R_i = 2.1 \pm 0.1 \) \( \mu \)m, near designed values that are 5x smaller than the mask radii, yielding a facial area of \( A_f = 10.0 \pm 0.2 \) \( \mu \)m\(^2\). SEM side views reveal a thickness of \( 1.0 \pm 0.1 \) \( \mu \)m. The measured average opening angle is \( \psi = 95^\circ \pm 1^\circ \), somewhat larger than designed. Optical diffraction also rounds the corners of the fabricated ASPs (radius of curvature for rounding \( \approx 200 \) nm); consequently, the measured \( \psi \) of particles is somewhat larger than designed.

2.2.3 Preparation of the Nanoparticle Dispersion

To form the 2D system, we mix a dilute dispersion of stabilized ASPs with a dispersion of a depletion agent composed of 42 nm diameter polystyrene (PS) spheres (carboxylate stabilized, surfactant free), yielding a final post-mixing ASP volume fraction of \( \phi \approx 0.03\% \), volume fraction of depletion agent \( \phi_d \approx 0.5\% \), and [SDS] = 5 mM.\(^{19-20}\) From the resulting mixed dispersion, a portion containing \( \approx 10^5 \) ASPs is loaded into a rectangular micro-cuvette (30 × 2.0 × 0.1 mm) and sealed using UV optical adhesive (Norland type 81). The diameter of the depletion agent has been chosen to lie between the smaller surface roughness on the faces of the ASPs and the larger surface roughness on their sides, thereby making it effective in inducing
anisotropic roughness-controlled depletion attractions.\textsuperscript{19} We have adjusted \( f_d \) to provide enough depletion attraction between the glass surface and a flat face of a particle, so that the particles lie flat on the glass substrate, effectively yielding a 2D monolayer of hard, Brownian ASPs. A short-range, Debye-screened electrostatic repulsion, having a screening length of only a few nanometers, (much smaller than the side-roughness), caused by adsorbed SDS, also exists between the flat faces of the particles and the lower glass surface, resulting in an aqueous lubricating layer between particles' faces and the proximate surface of the microcuvette. This repulsion also prevents in-plane aggregation of ASPs. Due to the lubricating layer, ASPs diffuse in the monolayer without lifting off or rotating out of the plane, even when a gravitational osmotic pressure is applied by tilting the microcuvette slightly. Although the edges of the ASPs are rougher than their faces, the contact potential associated with the in-plane depletion attraction between two ASPs is less than thermal energy, \( k_B T \), so ASP pair-interactions are effectively hard.

2.2.4 Characterization of a 2D Monolayer of ASPs

To explore the emergent self-organization of the 2D Brownian system of annular sectors, we tilt the cuvette along its length by an incline angle of \( \beta = 5^\circ \) to gravitationally concentrate the ASPs slowly and quasi-statically over three months, as shown in Figure 2.1c. After equilibration, annular sectors are imaged at different positions using a Nikon TE2000 inverted microscope equipped with a 40\( \times \) extra-long working distance objective lens (Nikon Plan Fluor ELWD, 0.6 numerical aperture), and a Nikon D5000 camera (4288 pixels x 2848 pixels). From a digital micrograph, the center coordinates of each annular sector are determined to a precision of about \( \pm 100 \) nm by fitting the outer arc to a circle.
2.3 Results

An optical micrograph of ASPs in an equilibrated 2D column is shown in Figure 2.1d. We assign a z-coordinate that points upward from a reference location in the dense part of column; by contrast, the depth, defined as $d = 240 \, \mu m - z$, is measured downward relative to a reference point above all particles, as shown. The total area fraction $\phi_A$ of ASPs increases for larger $d$, ranging from dilute at the top to concentrate toward the bottom. Gravitational forces effectively cause greater compaction of the ASPs deeper into the column, as reflected by the depth-dependent applied osmotic pressure $\Pi_{2D}(d)$. In the dilute region at the top of the column, single ASPs translate and rotate as individual monomers (Ms). However, for larger $d$, $\phi_A$ increases, and some monomers begin to interpenetrate and interlock with neighboring monomers to form dimers, which occupy space more efficiently. In this sub-surface region, which we refer to as a reaction zone, dimerization and de-dimerization reactions occur in equilibrium. If the tip of an arm of one ASP crosses an imaginary line connecting the two tips of the arms of a neighboring ASP, and vice-versa, then we consider these two mutually interlocking monomers to form one dimer. In 2D, the dimers are chiral objects, and we classify a dimer as being positive (D.+) or negative (D.-) according to the two possibilities shown in Figure 2.1d (inset). In the dense region at large $d$, the annular sectors are predominately in the form of dimers, with the exception of some scattered monomers and a single short chain; these represent defects in the dimer region.
Figure 2.1 Brownian microscale annular sector particles (ASPs), confined to a plane above the lower glass wall of a tilted optical cell, self-organize while diffusing in the presence of a gravitational osmotic pressure. (a) Schematic of an ASP as designed: $R_i$, $R_o$, and $\psi$ are its inner radius, outer radius, and opening angle, respectively. The black dot is the common center of the inner and outer circular arcs. (b) Scanning electron micrograph of a lithographically fabricated annular sector (scale bar: 1 $\mu$m). (c) Schematic of a tilted 2D gravitational column of annular sectors (black) after settling and equilibrating near one end of the optical cell. The tilt angle is $\beta$, and the depth into the gravitational column is $d$. OBJ: optical microscope objective lens (40x, extra long working distance). (d) Grayscale brightfield transmission optical micrograph after equilibration at $\beta = 5$ deg over 3 months. Each ASP has been color-coded as: monomer M (green), dimer($+$) $D_+$ (red), dimer($-$) $D_-$ (blue), and chain (yellow).
To interpret the osmotic pressure-driven formation of dimers from monomers in this near-equilibrium 2D Brownian system of ASPs quantitatively, we calculate its 2D osmotic equation of state $\Pi_{2D}(\phi_A)$. By filling ASPs in a grayscale micrograph with different colors using Photoshop and counting the colored pixels in a certain local bands (having a z-width of about 7.4 $\mu$m), we determine the area fractions of monomers, $\phi_{A,M}$, and dimers $D_\pm$, $\phi_{A,D\pm}$, as a function of $d$, as shown in Figure 2.2a. In the fluid-like phase at the top of the column, ASPs exist as monomers in the top of column, and a peak in $\phi_{A,M}$ is seen at a location $d_{M,\text{peak}} \approx 70 \mu$m. Just beyond this peak, $\phi_{A,D\pm}(d)$ increases exponentially and saturates into a plateau at larger $d$. Deep within the column, some scattered single monomers are completely encaged by dimers, and because of crowding, these monomers cannot reach other monomers to combine into dimers through quiescent thermal fluctuations. Consequently, $\phi_{A,M}$ fluctuates around a small value of about 0.02 even in the predominantly dimer region.

The peak in $\phi_{A,M}(d)$ can be empirically fit to a function composed of two exponential factors, each of which has a physical origin. A first factor, $[1+\exp(-(d-d_{0,M})/h_{g,M})]^{-1}$, describes an initial exponential increase arising from the applied osmotic gravitational pressure, which is given by a barometric law having a thermal-gravitational height for monomers, $h_{g,M}$.

A second factor, $[1+\exp((d-d_{0,M})/L)]^{-1}$, is associated with the exponential reduction of monomers deeper into the column and arises from a pressure-dependent near-equilibrium dimer formation reaction, where $L$ is a characteristic length scale and must be larger than $h_{g,M}$. Here, $d_{0,M}$ is a reference depth near the monomer peak that is common to the two exponential factors. The entire equation can be expressed as:

$$\phi_{A,M}(d)= \phi^0_{A,M} \left\{[1+\exp(-(d-d_{0,M})/h_{g,M})][1+\exp((d-d_{0,M})/L)]\right\}^{-1}$$

(2.1)
where $\phi_{A,M}^*$ is a scaling prefactor. Fitting the data, we obtain good agreement with a correlation coefficient of 0.93 [Fig 2.2a]; the values of the parameters are: $\phi_{A,M}^* = 0.55 \pm 0.06$, $d_{0,M} = 63 \pm 3 \mu m$, $h_{g,M} = 5.6 \pm 1.3 \mu m$, and $L = 16 \pm 3 \mu m$. The fit agrees very well with the peak, but the fitting function decays to 0 for larger $d$ and is not designed to account for residual trapped monomers within the dimer region where $\phi_{A,M}$ fluctuates around an average of about $\approx 0.02$ (dashed line).

For dimers, $\phi_{A,D\pm}(d)$ rises rapidly as the monomer concentration is depleted and then effectively saturates at large depths within our measured range. To fit this empirically, only a single factor is needed:

$$\phi_{A,D\pm}(d) = \phi_{A,D}^* \left[1 + \exp\left(-\frac{d-d_{0,D}}{h_{g,D}}\right)\right]^{-1}, \quad (2.2)$$

where $\phi_{A,D}^*$ describes a plateau value at larger $d$, $d_{0,D}$ is the critical distance of dimer, and $h_{g,D}$ is a characteristic length associated with the conversion of monomers to dimers by the gravitational osmotic pressure created by particles above the conversion layer. As shown in Fig. 2.2a, a fit using Eq. (2.2) to the measured $\phi_{A,D\pm}(d)$ yields excellent agreement (i.e. correlation coefficient = 0.998) and the following parameter values: $\phi_{A,D}^* = 0.370 \pm 0.003$, $d_{0,D} = 88 \pm 1 \mu m$, and $h_{g,D} = 9.4 \pm 0.5 \mu m$.

The total particle area fraction, $\phi_{A}(d)$, is shown in Fig. 2.2b and is described by the sum of Eqs. 2.1 and 2.2. We fix $h_{g,M} = 6.9 \mu m$ and $L = 19 \mu m$, using values obtained by fitting the monomer peak in $\phi_{A,M}(d)$. Because this monomer peak is effectively hidden in the initial rise of $\phi_{A}(d)$, these parameters cannot be independently extracted by fitting only $\phi_{A}(d)$ by itself. Fitting $\phi_{A}(d)$ to the sum yields: $\phi_{A,M}^* = 0.44 \pm 0.06$, $d_{0,M} = 68 \pm 1 \mu m$, $\phi_{A,D}^* = 0.401 \pm 0.002$, $d_{0,D} = 91 \pm 2 \mu m$, and $h_{g,D} = 15 \pm 1 \mu m$. These values are in reasonable agreement with those obtained by
fitting $\phi_{A,M}(d)$ or $\phi_{A,D\pm}(d)$ separately, within the overlap of standard deviations. Values of $\phi^*_{A,D}$ and $h_{g,D}$ are slightly higher when fitting $\phi_A(d)$; this small systematic deviation can be attributed predominantly to the trapped monomer contribution at large $d$ (i.e. the contribution given by the dashed line in Fig. 2.2a) that is not captured in the functional form of Eq. (2.1). Overall, all fits to $\phi_{A,M}$, $\phi_{A,D\pm}$, and $\phi_A$ agree well with the measurements, and their parameter values are self-consistent.

Using the measured $\phi_A(d)$, we calculate the applied gravitational 2D osmotic pressure, $\Pi_{2D}$, by summing up the effective buoyant mass of all particles above a particular height $z$:

$$\Pi_{2D}(z) = \left(\frac{k_B T}{A_p}\right) \int_z^\infty \phi_A(z')(dz'/h_{g,M}),$$

where $z'$ is a variable of integration along the $z$-direction.\textsuperscript{21}

After integrating, we convert $z$ to $d$, and we find that $\Pi_{2D}(d)$ begins to rise in the monomer region, and then rises more rapidly at larger $d$ beyond the monomer peak in $\phi_{A,M}(d)$ [Fig. 2.2b - right axis].

Thermal excitations in the Brownian system of ASPs subjected to a slow 2D osmotic compression can lead to dimerization of a pair of neighboring ASPs into a mutually interlocking configuration; a dimer pair can later de-dimerize into two monomer ASPs as a consequence of the same thermal excitations. Thus, the transient formation and dissociation of dimers is a dynamic process that is near equilibrium, and we hypothesize that this colloidal equilibrium reaction can be captured by the thermodynamic law of mass action.\textsuperscript{1} The chemical equilibrium equation describing dimerization and de-dimerization reactions is simply $2M \rightleftharpoons D_{\pm}$, as illustrated in Fig. 2.2c(inset). The equilibrium constant, $K$, of the dimerization reaction is given by the law of mass action: $K = \phi_{A,D\pm}/\phi_{A,M}^2$. We plot $K$ using the measured $\phi_{A,M}(d)$ and $\phi_{A,D\pm}(d)$, and we choose $K = 1$ where $\phi_{A,M} = \phi_{A,D\pm}$, at $d^* = 81.7$ $\mu$m, as determined by interpolation [see Fig. 2.2c].
Below the dilute surface fluid of monomers, in the active monomer-dimer reaction layer given by $70 \text{ \mu m} < d < 125 \text{ \mu m}$, $K(d)$ increases exponentially and can be fit well to:

$$K = \exp\left[\left(\frac{d-d^*}{L_D}\right)\right],$$

(2.3)

where $L_D = 6.49 \pm 0.01 \text{ \mu m}$ is a characteristic entropic-osmotic length scale associated with dimer formation. The exponential growth in $K$ shows that neighboring monomer ASPs efficiently convert into local dimer configurations when the applied osmotic compression begins to exceed $\Pi_{2D}(d^*) \approx 2k_B T/A_p$. 
Figure 2.2 Dependence of the particle area fraction $\phi_A$, osmotic pressure $\Pi_{2D}$, and equilibrium constant $K$ on the distance $d$ from the top of the tilted 2D column. (a) Area fraction of the monomers ($\phi_{A,M}$, black circles) and dimers ($\phi_{A,D\pm}$, blue squares). Dashed line: average $\phi_{A,M}$ over $125 \mu m \leq d \leq 240 \mu m$. Solid lines: fits to $\phi_{A,M}(d)$ using Eq. 1 and $\phi_{A,D\pm}(d)$ using Eq. 2.2 (see text); these fits intersect at $d = 81.7 \mu m$. (b) Total area fraction $\phi_A = \phi_{A,M} + \phi_{A,D\pm}$ (left axis). Solid line: fit by summing Eqs. 2.1 and 2.2. Two-dimensional osmotic pressure $\Pi_{2D}$ calculated from $\phi_A(d)$ by integration, normalized by $k_BT/A_p$, where $T$ is temperature and $A_p$ is the area per particle (right axis). (c) Equilibrium constant $K$, determined from the law of mass action of a monomer-dimer reaction using $\phi_{A,M}$ and $\phi_{A,D\pm}$ as a function of $d$. Here, $K = 1$ when equal populations of monomers and dimers coexist (i.e. where $\phi_{A,M} = \phi_{A,D\pm}$). Solid line: fit using Eq. 2.3. Inset: schematic of the equilibrium dimerization reaction: $2M \rightleftharpoons D_{\pm}$. 
The 2D osmotic equation of state, $\Pi_{2D}(\phi_A)$, of ASPs is calculated by eliminating the common parameter $d$ in $\phi_A(d)$ and $\Pi_{2D}(d)$, and normalized by the 2D thermal energy density $k_B T / A_p$ as shown in Figure 2.3a. Over a wide range of $\phi_A$, the scaled 2D osmotic equation of state can be fit to the semi-empirical form describing a crossover from an ideal-gas law to a jamming divergence:\(^\text{21}\)

$$\Pi_{2D}(\phi_A)/(k_B T / A_p) = f \phi_A/(1 - \phi_A/\phi_{A,c}), \quad (2.4)$$

where $f$ is a dimensionless prefactor and $\phi_{A,c}$ is a critical area fraction, corresponding to a divergence in $\Pi_{2D}$ at a jamming point. The fit accurately describes the data up to $\phi_A \approx 0.38$, yielding $f = 1.79 \pm 0.03$ and $\phi_{A,c} \approx 0.455 \pm 0.001$. For reference, an ideal gas of point-like particles would have $f = 1$; ASPs are not point-like, so a value of $f$ near unity is reasonable. Interestingly, the 2D osmotic equation of state does not reveal any abrupt features, which could possibly result from the underlying dimerization reaction, so the progression from predominately monomers to predominately dimers is gradual.

We also express the measured probability, $P_D$, of an ASP being found in a dimer configuration as a function of $\phi_A$ [see Figure 2.3a-inset]. For $\phi_A < 0.2$, towards the dilute limit, the system is predominantly populated by monomers: $P_D < 0.1$. However, in the monomer-dimer reaction zone, for $0.2 < \phi_A < 0.3$, monomers and dimers coexist in different proportions and $0.1 \leq P_D \leq 0.9$. In the highly concentrated regime for $\phi_A > 0.3$, long-lived dimers predominate $P_D > 0.9$, yet a small fraction of monomers becomes isolated from other monomers, so they cannot react and therefore become trapped.

The intrinsic nature of the pressure-driven dimerization reaction in the Brownian system of hard ASPs can be seen most obviously by plotting the natural logarithm of the dimerization
equilibrium constant, \( \ln K \), vs \( \Pi_{2D}/(k_BT/A_p) \) [see Figure 2.3b], where data are limited to the monomer-dimer reaction zone. A linear fit yields a slope of \( 2.7 \pm 0.2 \), implying that the number of accessible monomer microstates dramatically decreases as the applied \( \Pi_{2D} \) is raised near and above the entropic level \( (k_BT/A_p) \).

**Figure 2.3 Osmotic equation of state and equilibrium constant \( K \) of ASPs.** (a) Osmotic equation of state of ASPs, given by the scaled 2D osmotic pressure \( \Pi_{2D}/(k_BT/A_p) \) as a function of total area fraction \( \phi_A \). Solid red line: fit to \( f\phi_A, c\phi_A/(\phi_A, c - \phi_A) \); see text. Inset: probability of dimerization, \( P_{D+} \), as a function of \( \phi_A \). (b) Equilibrium constant \( K \) increases exponentially as a function of \( \Pi_{2D}/(k_BT/A_p) \). Solid line: linear fit to \( \ln K \) vs \( \Pi_{2D}/(k_BT/A_p) \), yielding a slope of \( 2.7 \pm 0.2 \).

In order to characterize the structure of ASPs at high density in the dimer region, we calculate the spatial pair correlation function \( g(r/D_o) \),\(^{28,29}\) where \( r \) is the separation between centers of two ASPs and \( D_o = 2R_o \) is the outer diameter of an ASP, for \( 0 \leq z \leq 88 \mu m \), as shown in Figure 2.4a. Interestingly, \( g(r/D_o) \) shows a strong discrete peak located at an unusually small value of \( r/D_o \approx 0.32 \), signaling the interpenetration of the ASPs that are dimerized as either D. or D. Thus, we define \( r_d \approx 0.32D_o \) to be the most probable center-to-center separation of dimerized ASPs in that region. For larger separations, we find strong peaks at \( r/D_o \approx 1.1 \) and \( r/D_o \approx 1.4 \), corresponding to pairs of ASPs that are in nearest neighboring dimers. Other smaller peaks are
found at larger $r/D_0$, but these only indicate short-range order, and overall the approach of $g$ towards unity indicates that the system is spatially disordered at long-range.

To explain the origin of the two local structural peaks in $g(r/D_0)$ beyond the dimer peak, we calculate $g(r/D_0)$ for a small idealized dimer crystallite composed of dimers having only a single chirality, based on non-overlap of ASPs, where the equilibrium configuration is an oblique lattice pattern having two non-orthogonal basis vectors of lengths $u_1$ and $u_2$. Closely spaced pairs of dots correspond to dimers, and we draw $u_1$ and $u_2$ between the centers of these pairs (see Fig. 2.4a: inset). By studying different inter-particle distances within the dimer crystal, we conclude that the peak near $r/D_0 \approx 1.1$ is related to packing of circles at a density, corresponding to the distance $u_1$, that is somewhat below close packing. Additional contributions to this peak come from shorter diagonals between ASPs in neighboring dimers in a 4-dimer local unit cell. The peak near $r/D_0 \approx 1.4$ results from the longer diagonal distances between ASPs in the same 4-dimer local unit cell. Although the disorder present from the racemic mixture, as well as monomer defects, in the experimentally observed dimer region destroys order at long range, the crystal model is still useful in explaining non-dimer peaks arising from local order in $g(r/D_0)$. The ring-like patterns that decay towards higher wavenumbers in the Fourier transform of the real-space monochrome micrograph ($62 \leq x \leq 152 \ \mu m$, $0 \leq z \leq 90 \ \mu m$) is also an indication of short-range order, as shown in Figure 2.1c. Some degree of azimuthal asymmetry is seen in the Fourier transform towards lower wavenumbers, indicating that there can be local orientational order between dimers composed of ASP monomers. This Fourier transform pattern is interesting because it represents a combination of interference effects that would be seen in a scattering experiment from the experimentally observed system; this combination includes interference effects between interpenetrating ASPs within dimers as well as interference effects between
dimers with other neighboring dimers. The separation of a Fourier transform into the product of a form factor and a structure factor, which is appropriate for many molecular and colloidal systems, is not appropriate in this case because of the interpenetrating nature of the lock-and-key dimers.

Figure 2.4 Spatial pair correlation function and FTs when ASPs become dimers. (a) Spatial pair correlation function $g(r/D_0)$ calculated from the lower part ($0 \leq z \leq 88 \, \mu m$) of the micrograph shown in Fig. 2.1d. The center-to-center pair separation, $r$, is normalized by outer diameter, $D_0$, of the ASPs. Inset: oblique unit cell of a perfect $D_+$ dimer crystal (unit vectors: $u_1$ and $u_2$). Each dimer is given by a pair of black dots (representing the centers of two ASPs) separated by $r_d$. (b) Fourier transform of the dense dimer region ($62 \leq x \leq 152 \, \mu m$, $0 \leq z \leq 90 \, \mu m$) of the micrograph shown in Fig. 2.1c. White scale bar: $2 \, \mu m$.

2.4 Discussion

We have revealed nearly perfect sub-surface dimerization of ASPs into a dense, spatially disordered phase below a fluid layer at the surface which is predominantly composed of monomer ASPs. Since the ASPs have only hard interactions, their free energy is entirely determined by entropy and geometry, and the local arrangement of ASPs into dimers essentially is a byproduct of more efficient local organization that allows a larger number of translational
and rotational microstates for all particles in the system. Thus, dimerization of hard ASPs by an applied osmotic pressure is different than well-known dimerization in systems of attractive monomers. It is interesting and striking that a form of local chiral symmetry breaking (LCSB)\textsuperscript{22} occurs in each dimerization event, and the product dimers are found in racemic proportions, consistent with the achiral nature of individual ASPs. D\textsubscript{+} and D\textsubscript{-} are mirror-images, so they are enantiomeric chiral objects that have similar incommensurate edge lengths. Random dimerization during the slow compression of the system leads to spatial disorder even in the presence of Brownian fluctuations. Since there is some short-range orientational order among neighboring dimers, the system could be interpreted as being a polydomain liquid crystal\textsuperscript{29} made up of very small domains, containing a scattering of monomer defects, the vast majority of which cannot easily recombine. Regardless of whether the system is regarded as a polydomain liquid crystal or a disordered glass, the dense racemic system of D\textsubscript{+} and D\textsubscript{-} is highly disordered at medium and long range, and it exhibits non-ergodic dynamics.

Although we have achieved a very high percentage of dimerization in the dense phase below the primary sub-surface reaction zone, a few monomers, nearly all of which are isolated, still remain. Such isolated monomers are prevented from diffusing into proximity of other monomers by the presence of many dimers in between. The probability of monomers moving by a series of successive de-dimerization and re-dimerization events to finally recombine is also not entropically likely, since considerable local work must be done against the osmotic pressure for even a single de-dimerization event to occur. In the reaction zone, it is possible for an odd number of ASPs in a local region to form as many dimers as possible, leaving only one ASP unpaired, simply as a consequence of the discrete nature of the building blocks. Since the reaction zone can be divided into many local regions, monomers in each region can be separated
from each other, and their transport properties within the reaction zone relative to the rate of compression will largely control the density of residual isolated monomers found in the dense dimer region below. These monomers are effectively point-like defects trapped in the dense dimer region, although they can still rotate.

2.5 Conclusions

In conclusion, we have performed experiments on Brownian systems of hard annular sector particles that reveal a high degree of dimerization. By investigating a very slow 2D osmotic compression of a Brownian monolayer of ASPs using optical microscopy, we have shown that the equilibrium constant \( K \) of the dimerization reaction grows exponentially as \( \Pi_{2D} \) increases. Moreover, we have revealed that the dense dimer region is predominantly composed of racemic populations of chiral enantiomers \( D_+ \) and \( D_- \), a form of local chiral symmetry breaking that ultimately promotes disorder in the system. While individual ASPs are not bonded via attractive interaction into dimers, nevertheless, lock-and-key dimers of hard ASPs are well-defined, topologically identifiable structures, and these dimers can be treated as composite units according to principles of equilibrium thermodynamics governing composition and decomposition reactions.

Our exploration of this system of ASPs leads to a number of exciting future directions. Although we have demonstrated robust dimerization of a single ASP shape, a broader theoretical question still remains about how to generally describe all necessary characteristics of the entire range of shapes of hard particles that would give rise to robust dimerization, while excluding other potential local polymorphs, in a Brownian system driven by a slowly applied osmotic pressure. It is possible that increasing the size of the reaction zone, for example by decreasing the
tilt angle of the cuvette, could further reduce the already small monomer population in the dense dimer region. In addition, directly measuring the forward and reverse reaction rates in the reaction zone, by taking and analyzing time-lapse video recordings, could lead to a real-space method of determining $K$ for colloidal reactions through the principle of detailed balance. Finally, theoretically calculating the number of accessible microstates for ASPs or performing molecular or Brownian dynamics simulations at different densities could be used to predict the behavior that we have measured and generalize these results.
References


Chapter 3 – Dimer Crystallization of Chiral Proteoids

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3.1 Introduction

High resolution structures of many proteins have been obtained by combining protein purification and crystallization\(^1\text{-}^4\) with coherent x-ray scattering, electron scattering, and nuclear magnetic resonance\(^5\text{-}^7\). Many crystallization protocols exist and perform well for limited classes of proteins\(^8\text{-}^9\), yet no single universal procedure can robustly crystallize all proteins, particularly membrane proteins\(^10\). Genetic mutations and misfolding can also alter primary and secondary structures of proteins, and, this in turn, influences their capacities to crystallize and affects their resulting crystal structures\(^11\text{-}^{12}\). For instance, changes in morphologies of amyloidogenic proteins have been linked to Alzheimer's disease\(^13\text{-}^{14}\). Given the importance of protein crystallization, developing a clear understanding about the basic mechanisms is therefore pressing, yet key questions about protein crystallization and more broadly self-assembly remain unanswered.

As examples, the regulatory protein, Cro, from bacteriophage lambda\(^15\) and the coat protein of bacteriophage MS2\(^16\), have been self-organized to the quinary structural level\(^17\), yielding ordered arrays of homodimers\(^18\). Thus, the crystallization of systems of nanoscale proteins represents an important example of the hierarchical self-assembly of naturally occurring colloids. Moreover, the extent to which entropy and the repulsive core geometry of proteins contribute to their crystallization, compared to other effects, such as site-specific attractive
interactions including hydrogen bonding, has not been clearly quantified experimentally. Consequently, to better understand the mechanisms associated with the crystallization of complex colloids, including proteins, that have nontrivial shapes and are continuously subjected to fluctuating entropic forces, it would be reasonable to examine the impact that the simpler combination of entropy and hard-core shape can have on colloidal self-assembly in crowded environments at high densities.

In an exciting development, microcrystals of proteins have been identified using electron microscopy; electron scattering multi-angle 3D tomography has been performed on an individual microcrystal, thereby significantly reducing the overall crystal size that is required to determine its structure\textsuperscript{19}. Thus, even the production of relatively small crystallites by limited protocols can still yield important information about certain protein structures and crystals. This direct real-space approach is particularly appealing if limited quantities of building-blocks, such as purified proteins, are available. Moreover, this approach is reminiscent of prior real-space optical microscopy experiments on colloidal crystals of hard lithographic particles, such as squares, rhombs, crosses, and tri-stars, that have been crowded and in which microcrystals have been observed and structurally characterized\textsuperscript{20-23}. For such relatively simple shapes, the dominant crystalline polymorph has been quantitatively explained using the concept of entropomorphism: maximization of the combined translational and rotational entropy controls the crystal type as well as the average relative positions and orientations of individual objects within it\textsuperscript{23}.

In living biological systems, proteins often fold and function in highly crowded aqueous environments. This crowding can cause the core shape to play a very significant role in how a protein interacts with other proteins around it. Moreover, proteins are subject to ever-present entropic thermal excitations that cause them to explore physically accessible microstates, so
entropy can also play an important role in crowded systems of many proteins. Most protein crystallization protocols involve slow evaporation of a purified protein solution, which increases protein crowding and also can increase the concentrations of osmolytes present in the aqueous environment. In certain cases, when a single protein of interest does not readily crystallize, its structure can sometimes still be determined by adding a chaperone protein that dimerizes with it\(^{24-26}\). The resulting protein heterodimers more readily form crystals, from which their atomic structures can be extracted, typically by x-ray diffraction. Thus, dimerization is often a desirable prerequisite that can enhance ordering of certain proteins into crystals.

Mutations, misfolding, and denaturation are mechanisms that can cause proteins to change shape, alter local biomolecular interactions, and influence self-assembled structures\(^{12,27-28}\). In particular, purposefully introducing genetic mutations can be useful for facilitating their crystallization of certain proteins\(^{29-30}\). The introduction of genetic mutations, either through natural processes or human intervention, can lead to proteins that have different non-standard phenotypic shapes after translation and folding. In some cases, the resulting structural changes can be deleterious and lead to disease; in other cases, they could be potentially beneficial.

By contrast, here, we directly mutate and express a precisely desired phenotypic shape, including its chirality, with a high degree of replication using top-down lithography. Moreover, we explore the concept of lithographic mutations of highly precise proteomimetic shapes and the impact that such mutations can have on the resulting self-organized structures. A high degree of lock-and-key dimerization has been achieved in Brownian two-dimensional (2D) systems of achiral annular sector particles (ASPs), resembling "C"-shapes, which become more crowded as an osmotic pressure is slowly applied\(^{31-32}\). When two monomer ASPs dimerize, two distinct reaction pathways are possible yielding either positive chiral dimers (D\(_+\)) or negative chiral
dimers (D.) (Figure 3.1a). For a random system of many monomers that are concentrated, racemic 50:50 production of D. and D. suppresses ordering of the dimers. Thus, although the achiral ASP shape does facilitate a high degree of dimerization, because the dimerization pathway is not selective, the resulting racemic mixture of dimers remains disordered.

Although achiral ASPs do not form ordered dimer crystals when crowded, if one could force the dimerization pathway to only select either D. or D., for instance by steric suppression, then the enantiopure dimer system could have a much greater proclivity to crystallize, reminiscent of chiral protein crystals composed of chiral dimers. Recognizing that many proteins are chiral and some have shape-complementary protrusions and receptacles (e.g. clefts, crevices, or cavities), we mutate the shape of achiral ASPs into proteomimetic chiral shapes by putting a head on only one of the two protruding arms, ensuring that the arm chosen is the same for all particles, thereby creating an enantiopure chirality of all printed particle shapes. We have chosen the size and location of this head, a sub-structure which represents a relatively small change to the overall perimeter and area compared to an achiral ASP, to inhibit the dimerization pathway for D. completely, so that any dimers that form through crowding will be enantiopure D. (Figure 3.1b). We design and explore several different proteoid shapes that sterically enforce enantiopure chiral pathway selection (CPS), in which the placement of the head relative to the body of the annular sector is head-interior (HI), head-centered (HC), and head-exterior (HE) (Figure 3.1c,d). Thus, lithographically shape-designed proteomimetic colloids, or "proteoids", when crowded, potentially could offer a route to dimer crystallization by hierarchical self-assembly in which entropy and hard-core shape play essential roles.
**Figure 3.1 Shape-designed proteomimetic chiral colloids.** (a) Reaction scheme of achiral ASPs leads to racemic dimerization into isomers D+ and D−, which frustrates crystallization. (b) Reaction of two chiral proteoids leads to enantiopure dimerization into D+ only, since head-mutations sterically inhibit pathways to D−. (c) Designed shapes of proteoids (from left to right): head interior (HI), head centered (HC), and head exterior (HE). (d) Scanning electron micrographs of lithographically fabricated HI, HC, and HE proteoids composed of crosslinked SU8 photoresist. Scale bar = 1 µm.

Excellent control over the chirality of Brownian lithographic parallelograms in monolayers has been recently demonstrated through the development of facially anisotropic roughness controlled depletion attractions (FA-RCDA)\textsuperscript{33-34}, a specific application of shape controlled depletion attractions\textsuperscript{35}. Thus, a dispersion of chiral lithographic proteoids, when mixed with a suitable depletion agent (e.g. polystyrene nanospheres) and sealed into a rectangular optical microcuvette, can yield an enantiopure 2D Brownian monolayer of mobile proteoids that
have effectively hard in-plane interactions (see Materials and Methods section in this chapter). Obtaining precise chiral control over the monolayer is non-trivial and requires an enantioselective method such as FA-RCDA. Otherwise, chiral particles that settle to the lower wall of the microcuvette would randomly deposit, such that either of their two faces would have equal chances of being near the lower wall, thereby leading to the formation of a racemic monolayer. Such an approach would facilitate highly controlled experimental realizations of a wide variety of lock-and-key interactions, some of which have been studied theoretically\textsuperscript{36}.

3.2 Materials and Methods

3.2.1 Lithographic fabrication of proteoids

Solid microscale particles composed of epoxy photoresist SU-8 (Microchem Inc.) are fabricated using top-down photolithography, yielding a stable aqueous dispersion of monodisperse polymeric prismatic platelets that have customizable shapes\textsuperscript{37}. Proteoids composed of SU-8 polymer photoresist, having a uniform thickness of 1 µm, are fabricated on four inch diameter wafers using top-down photolithography (ASML stepper, PAS 5500, i-line 365 nm ultraviolet (UV) light), released by dissolving a 120 nm thick sacrificial layer of LOR-1A (Microchem Inc.) using an aqueous solution of 40 mM sodium dodecyl sulfate (SDS, MP ultrapure) and 1.8% w/v tetramethylammonium hydroxide (TMAH, Sigma-Aldrich, 25% in water). Adsorbed dodecyl sulfate anions on the surfaces of the particles inhibit particle aggregation. We concentrate and wash the aqueous dispersion of proteoids by gentle and brief centrifugation, remove the supernatant containing residual dissolved LOR and TMAH, and then dilute the particles with a 5 mM aqueous SDS solution. To further reduce the concentrations of
LOR and TMAH, we repeat this washing procedure 3 more times, yielding a stable aqueous dispersion of \( \approx 200 \) million monodisperse proteoids per wafer in a 5 mM SDS solution.

### 3.2.2 Forming Dense Monolayers of Brownian Proteoids

To a dilute aqueous dispersion of proteoids, we add a dispersion of a nanoscale depletion agent (Invitrogen, 42 nm diameter polystyrene spheres, carboxylate stabilized, surfactant free), yielding a post-mixing proteoid volume fraction of \( \phi \approx 0.03\% \) and a post-mixing depletion-agent volume fraction of \( \phi_d = 0.5\% \). This depletion agent induces a facially anisotropic roughness-controlled depletion attraction (FA-RCDA)\(^{34}\). We load the mixed dispersion containing \( \sim 10^6 \) proteoids into a rectangular optical cuvette (30 mm \( \times \) 2.0 mm \( \times \) 0.1 mm), place the loaded cuvette flat in the center of a standard glass microscope slide, and seal both open ends with UV optical glue (Norland, type 81). The FA-RCDA causes a preferential attraction of only one face of each chiral ASP towards the lower flat smooth wall of the optical cuvette. After \( \approx 10 \) hours of sedimentation while horizontal, proteoids are observed to diffuse translationally and rotationally in this dilute enantiopure monolayer. Screened electrostatic repulsions, arising from the adsorbed SDS, prevent the proteoids from irreversibly adhering to the lower wall and preserve lubrication between the proteoids and the lower wall even in the presence of the depletion attraction. The diameter and post-mixing \( \phi_d \) of the depletion agent have been optimized to induce FA-RCDA that lead to \( > 99.9\% \) enantiopure monolayer formation of proteoids, as determined by examining more than 1,000 proteoids that diffuse above the flat surface in the presence of FA-RCDA.

In order to slowly concentrate the particles and enable crystal formation without substantial non-equilibrium jamming of particles that can lead to disorder, we tilt the cuvette along its long axis at a small angle of 5° and monitor the slow sedimentation towards the lower
end of the cuvette. After a period of three months, during which the particles become gradually crowded towards this lower end, we then image the column from the top to the bottom using an inverted microscope (Nikon TE2000) equipped with a 40× extra-long working distance objective lens (Nikon Plan Fluor ELWD, 0.6 numerical aperture) and a Nikon D5000 camera. Additional detailed information regarding Materials and Methods, including image analysis methods, are provided in Appendix A at the end of Chapter 3.

3.3 Results

A microcuvette containing fluctuating chiral(+) HI-proteoids (Figure 3.1a-d) in a dilute enantiopure monolayer (> 99.9%) is slightly tilted by 5 degrees, causing them to crowd slowly (see Materials and methods), as a consequence of an applied 2D gravitational osmotic pressure. As they sediment towards one end of the microcuvette, their area fraction, \( \phi_A \), there gradually increases and approaches a steady state. Using brightfield transmission optical microscopy (Nikon TE2000, tilted 5 deg, 40x objective), we record time-lapse movies as well as high resolution static images of the resulting column at different locations, taken after a very slow compression over three months (Figure 3.2a and Figure 3.3).

**Figure 3.2 Slowly crowding Brownian HI-proteoids in a monolayer leads to their hierarchical self-organization into enantiopure dimer crystals.** (a) Hard proteoids diffuse in a Brownian monolayer just above a flat smooth glass plate, as observed using optical microscopy (objective lens: OBJ). The plate is tilted by a small angle \( \beta \), causing the proteoids to concentrate slowly towards the lower end. (b) Schematic of local arrangements of HI-proteoids in two distinguishably different chiral crystal forms: HI-DX1 and HI-DX2.
At the top of the column, we observe a dilute gas-like region, in which monomer proteoids exhibit relatively unconstrained translational and rotational Brownian motion. Any lock-and-key dimers formed in the gas-like region are short-lived and decompose into monomers shortly thereafter. Below this, a liquid-like layer exists at higher \( \phi_A \); in this layer, at any given instant, significant numbers of enantiopure dimers are seen in addition to monomers. Both dimerization and de-dimerization reactions occur frequently in this region, which we refer to as the dimerization reaction zone. Below this, we see a densely crowded region composed mostly of dimers, but also including a minor yet significant residual fraction of monomers. Moreover, the area fraction occupied by D+ dimers, \( \phi_{A,D^+} \), rises rapidly in the dimer reaction zone and then more slowly in the densely crowded region below (see Appendix A Fig. S3.1).
Figure 3.3 Overlapping optical micrographs are stitched together to provide an extended view of the column where proteoids have become crowded. A gas-like region at the top of the column is composed mostly of monomers (green). Below this, a dimerization reaction zone is seen; all dimers (red) formed have only one chirality. Deeper into the column, micro-crystallites of two different chiral crystal types, HI-DX1 (yellow) and HI-DX2 (light blue) are found. The depth $d$ is defined from the reference shown. Dotted black line: stitching boundary of two micrographs. Black dashed lines: grain boundaries between dimer crystallites. Scale bar (upper right): 10 $\mu$m.
Within this densely crowded region, we also observe crystallites of two different types of dimer crystal configurations, HI-DX1 and HI-DX2, which both have the same positive chirality of the constituent monomers and dimers. Through a sequence of dilation, rotation, translation, and compression of all dimers, HI-DX1 can be transformed into HI-DX2, and vice versa (Figure 3.2b). For instance, if the long axes of the dimers in DX1 are rotated by a relative angle of about 40°, then these dimers most naturally interdigitate in a different crystal configuration: DX2. For such rotations to occur in the dense system, a local collective fluctuation in $\phi_A$ is necessary to create a temporary local dilation. Although DX1 is more frequently seen than DX2 deep in the tilted column, the entropies of these two crystal configurations are very similar, so we still see a substantial proportion of DX2. As a consequence of dimerization, the first stage in hierarchical assembly of dimer crystals, concave and convex corrugations emerge in the overall perimeter shape of D+. These corrugations facilitate the lock-and-key alignment of dimers into the two crystal types. Protrusions of one dimer fit into complementary indentations of a neighboring dimer, thereby creating dimer-dimer interdigitation. For HI-DX2, the head of a monomer in one dimer is immediately opposite and pointing toward the head of a monomer in a neighboring dimer; by contrast, in HI-DX1, nearby heads point away. The primitive vectors between the centers of nearest neighboring dimers for HI-DX1 are $|a_1| = 5.2 \pm 0.1 \mu m$ and $|a_2| = 8.2 \pm 0.2 \mu m$ with a tilt angle with respect to the $x$-direction of $\gamma = 72^\circ \pm 1^\circ$; for HI-DX2, these are $|a_1| = 5.6 \pm 0.1 \mu m$ and $|a_2| = 8.2 \pm 0.2 \mu m$ with $\gamma = 57^\circ \pm 1^\circ$. Much further below the dimer reaction zone, where the crowding is higher and self-assembly times at high densities are effectively longer, we find larger microcrystallites of HI-DX1 as well as a minor fraction of significantly smaller HI-DX2 configurations surrounded by an amorphous mixture of monomers and dimers. However,
the smallest crystallites of HI-DX1 and HI-DX2 appear at a minimal area fraction of about 0.37 ± 0.02 near the top of the column. There may be a difference in the minimal area fraction between these two types of crystals, but this difference is smaller than our uncertainty in determining the area fraction. Interestingly, monomers are often seen in higher-than-average proportions at the outer edges of growing crystallites, compared to the bulk amorphous mixture, potentially linked to their expulsion from the cores of these crystallites.

Isolating a large microcrystal of HI-DX1, we retain its ordered core, digitally fill the regions inside all proteoids uniformly in black and regions outside them in white, and rotate the result to align a primary crystal axis with the x-direction, as shown in Figure 3.4a. By taking the square root of the sum of the squares of real and imaginary parts of the complex fast Fourier transform (c-FFT) in Matlab\(^{38}\), we calculate the magnitude of the complex field (Figure 3.4b); the square of this magnitude would ordinarily only be available as an intensity scattering pattern measured using scattering techniques (see Appendix A). We also determine the real and imaginary parts of the scattering field, shown in Figure 3.4c,d, respectively, that clearly reveal Bragg peaks caused by the ordered lock-and-key chiral dimers. Some peaks in the real part of the c-FFT are much more prominent than in the imaginary part at the same wave vector whereas others are missing, and vice-versa. This is a consequence of interference effects caused by interlocking of monomers, shape-complementary corrugation matching of adjacent dimers, and the overall chiral nature of the monomers, dimers, and crystal itself. Current x-ray scattering methods are unable to measure the real and imaginary parts of the field scattered from protein crystals in reciprocal space, so modeling is often used to deduce missing phase information\(^{39}\). By contrast, here we provide examples of real and imaginary scattering patterns of dimer crystallites.
that portend similar results for protein crystals if coherent x-ray scattering techniques can be developed for directly measuring the phase of the field, not just its amplitude, in reciprocal space.

Figure 3.4 Structure and Fourier transforms of an isolated self-assembled chiral dimer crystallite (DX1) of chiral HI-proteoids. (a) Real space optical microscope image: proteoids have been filled to a uniform black density and the space outside is uniformly white. Scale bar: 3 µm. HI-DX1 is an oblique lattice; lengths of average primitive vectors (red arrows) between dimers are: |\(a_1| = 5.2 \pm 0.1 \text{ µm}, |\(a_2| = 8.2 \pm 0.2 \text{ µm};\) internal angle \(\gamma = 72° \pm 1°.\) Particle area fraction of dimer crystal: \(\phi_A \approx 0.47.\) (b) Common scattering pattern, expressed as the field amplitude in reciprocal space, resulting from a Fourier transform of the HI-DX1 crystallite in (a). (c) Real and (d) imaginary parts, respectively, of the complex Fourier transform of (a). Constructive and destructive interference effects differ in the real and imaginary scattering patterns, which can be converted to amplitude and phase. White scale bars are 3 µm\(^{-1}.\) Fourier intensities are in arbitrary units after converting the image to double precision.

Exploring the kinetics of crystal formation below the reaction zone using time-lapse video microscopy, we find that random Brownian motion can lead to multi-particle reactions that effectively facilitate monomer movement. We call these reactions tautomerization translocation.
reactions (TTRs). A lone monomer proteoid can react with a neighboring dimerproteoid in following sequence: the dimer proteoid decomposes into two monomers, one of which reacts with the initially lone monomer to form a dimer, causing the other to become a new lone monomer that is significantly displaced from the original lone monomer (Figure 3.5a,b). This mechanism expedites the expulsion of monomers from the interiors of crystals that are forming. It also increases the rate of dimerization of monomers that would otherwise remain trapped and isolated in the dense region. Without TTRs, which are direct consequences of rotational and translational diffusion of individual proteoids as well as their collective Brownian density fluctuations, the long-time diffusion coefficient (proportional to the slope of the monomer's mean square displacement) of a monomer encaged by dimers at high $\phi_A$, would effectively be zero. Thus, TTRs effectively provide mechanisms for long-time monomer migration in the dense system, even as individual proteoids can exchange roles as monomers and dimers without translating very far. The largely circular nature of the outer edge of monomer HI-proteoids facilitates their rotational diffusion in the dense system without as much interference as would be experienced by anisotropic, non-circular objects. This capacity of monomers to diffuse rotationally in the dense system enables them to align with neighboring dimers more frequently and increases the rate of this TTR. A second collective tautomerization reaction, which involves four proteoids, two monomers and one dimer, can also effectively facilitate the displacement of both monomers without requiring much change in the local density (Figure 3.5c,d). Overall, both TTRs increase the rate of monomer transport and facilitate annealing within the dense system, leading to an effective diffusion coefficient for monomers that is much higher than what is typical in dense 2D systems of other shapes. This higher monomer transport rate facilitates dimer crystallization even in highly crowded environments.
Figure 3.5 Collective tautomeration translocation reactions (TTRs) in dense Brownian systems of chiral HI-proteoids. (a) Schematic of a monomer-dimer proteoid TTR in which a monomer effectively moves through a de-dimerization and dimerization reaction. Proteoids in dimer configurations are in red; monomers are in green. Numbers label the proteoids, which remain distinguishable at sufficiently high frame rates. (b) Example of an observed monomer-dimer TTR of HI-proteoids. Middle and right frames are 24 min and 27 min after the left frame. (c) Schematic of a 2-monomer-dimer proteoid TTR. Two monomers effectively move as a consequence of dimerization and de-dimerization events. (d) Example of an observed 2-monomer-dimer TTR. Middle and right frames are 3.5 h and 18.5 h after the left frame. Scale bar: 5 µm.

Beyond dimerization reactions and TTRs, a wide variety of dimer reconfiguration reactions can occur and are also important. For instance, in the presence of Brownian density fluctuations, dimers that are locally configured in HI-DX1 can transform by slight rotations and translations into HI-DX2 configurations. The prominence of HI-DX1 over HI-DX2 indicates that one crystal form has a subtle yet observable advantage over the other in yielding more accessible translational and rotational microstates, which is favored in terms of entropy maximization,
related to the number of translational and rotational accessible microstates of the collection of proteoids. Grain boundaries between neighboring crystallites are also observed, and these boundaries can also slowly evolve.

In effect, the lock-and-key dimers of proteoids can be thought of as compressible quasi-particles, even if individual monomer proteoids are hard objects. This effective compressibility of dimers facilitates large local density fluctuations which promote annealing during crystal growth even at high proteoid densities. These fluctuations can contain locally correlated proteoid motions, resembling the collective spatiotemporal heterogeneous dynamics found in glassy materials near the glass transition\textsuperscript{40}. In addition, these density fluctuations may be further amplified by the gradient in the concentration of proteoids in the tilted system, which may play a role similar to that of the gradient in temperature of traditional molecular systems\textsuperscript{41}.

Although the proteoids are quite rigid, as monomers combine into dimers, forward and backward monomer-dimer interconversion reactions can effectively create local density fluctuations reminiscent of systems of objects that have significant compressibility (Appendix A Figs. S3.1 and S3.2). These large local density fluctuations can cause dramatic heterogeneous dynamics, sometimes even resembling avalanches, involving the coordinated collective motion of many proteoids in a localized region. Such avalanches can cause local ordering of dimers, yet less frequently avalanches can also cause local disordering of dimers. Over very long time scales (\textit{i.e.} one month), time-lapse imaging reveals a very slow continuing compaction of all proteoids, indicating that monomer consolidation into dimers deep within the monolayer column is still occurring. Thus, the system is not strictly in equilibrium, even as large crystallites are forming. Local density fluctuations are frequent and large enough that they enable reconfigurations of individual proteoids and dimers even in a crowded environment, so the system does not become
trapped or jammed in a non-ergodic, disordered state. Crystallization is thereby facilitated even at high $\phi_A$, since entropic Brownian excitations drive a combination of large density fluctuations, near-equilibrium, forward and backward monomer-dimerization/de-dimerization reactions, TTRs, and other local reconfiguration reactions. This, in turn, creates an evolving free energy landscape that is effectively able to optimize the packing density in local regions through crystallite formation. Ongoing dimerization, local reconfiguration, and crystallization reactions in crowded fluctuating Brownian systems of proteoids create a continuously evolving non-equilibrium spatial density field as individual crystallites form and grow.

Beyond HI-proteoids, we have investigated two other proteoid shapes in which the head location, its relative size to the annular sector, and the resulting opening angle have been varied: head-centered (HC) and head-exterior (HE) (Figure 3.1c,d). We find that DX1 and DX2 crystals form for both HC and HE proteoids, yet the proportions and sizes of the observed crystallites are not exactly the same as for HI-proteoids in the crowded region. Examples of self-assembled dimer crystals formed by slowly crowding HC-proteoids are shown in Figure 3.6a,b, and examples for HE-proteoids are shown in Figure 3.6c,d. One apparent advantage that HI proteoids have over HC or HE is kinetic: rotational diffusion and reorientation of monomers in the crowded environment is more rapid because the exterior edge of the proteoid is closer to circular. By comparison, external heads and larger openings, which cause protrusions and higher asymmetry of the proteoids, can significantly inhibit the rotational diffusion of monomers in the high density regions of the monolayer, so the kinetic rates of crystallization of these shapes is therefore slower.
Figure 3.6 Self-assembled dimer crystal structures of chiral HC- and HE-proteoids. Insets: red arrows show primitive vectors of the dimer lattices. (a) Head-centered (HC) proteoid self-assembles predominantly into a rectangular lattice of HC-DX1 dimers. (b) HC-proteoids also form a secondary self-assembled HC-DX2 lattice that is oblique. (c) Head-exterior (HE) proteoids self-assemble in to an oblique dimer crystal HE-DX1 lattice. (d) Also observed are crystallites of dimerized HE-proteoids that have a different oblique structure, HE-DX2. Scale bar: 5 µm. Area fraction ϕ_A of dimer crystals: (a) and (b) 0.47; (c) and (d) 0.46. Average lengths of primitive vectors |a1| and |a2| (in µm), internal angles γ (deg): (a) 5.1 ± 0.1 and 8.4 ± 0.1, 90° ± 2°; (b) 6.5 ± 0.1 and 8.3 ± 0.2, 52° ± 2°; (c) 5.2 ± 0.2 and 8.0 ± 0.1, 84° ± 3°; (d) 6.8 ± 0.1 and 7.8 ± 0.2, 49° ± 2°.

A different aspect of the hierarchical self-assembly is the effect of corrugations that are found along the external edge of the self-assembled dimers; some degree of corrugation appears to be advantageous in crystallization, since it helps spatially order dimers that have their axes oriented along the same direction. Without such corrugations, the system might otherwise form an oriented liquid crystal of dimers that does not possess spatial order over a range that is many particle diameters. Slight changes in the geometries of the proteoids can strongly influence the sizes, structural types, and primitive vectors of the resulting dimer crystals.
For instance, HI-proteoids have the greatest propensity to form the largest dimer crystals out of all shapes we have examined so far; nevertheless, HC- and HE-proteoids, which have different shapes, can also readily form dimer crystals in monolayers that are slowly crowded. In Figure 3.6a,b, we show that the primitive vectors of two self-assembled lattices of HC-proteoids can be either rectangular or oblique. Thus, the fundamental space-group of a self-assembled dimer crystal can be changed only by introducing slight mutations in the shape of a proteoid (e.g. from HI to HC). The c-FFTs of DX1 and DX2 of HC-proteoids (Appendix A Figures. S3.3 and S3.4) are different than those of either HI- or HE-proteoids since the primitive vectors and orientations of dimers within these self-assembled crystals reflect differences in the individual proteoid shapes. Likewise, by shifting the head further towards the outer radius of the ASP and changing the opening angle, two different oblique lattices are found for HE-proteoids, as shown in Figure 3.6c,d. These oblique lattices of dimers of HE-proteoids have different primitive vectors than those of the dimer crystals of HI-proteoids.

3.4 Discussion

The phenomenon of dimer crystallization in crowded monolayers is observed to be general and robust with regard to limited changes in shape, provided a head is sized and placed in a manner that forces chiral pathway selection during dimerization. However, just as relatively small changes from an achiral ASP to a chiral proteoid through the addition of a head has a profound impact on the self-organization, the size and placement of this head and other features of the ASP shape, such as the opening angle, can also profoundly impact the basis vectors of the self-organized dimer crystal structure. In effect, the external shapes of self-assembled lock-and-key dimers can be designed to present regions of concavity and convexity to other dimers; these effective shapes of dimers are influenced by small changes in the shapes of the constituent
monomers. Shape complementary regions on neighboring dimers can thereby also facilitate lock-and-key ordering of these dimers into crystals. This diversity of crystal structures that we have observed for proteoids having different core shapes is reminiscent of the diversity of crystal structures that are known among amyloidogenic proteins.\(^{\text{43}}\)

While lithographic proteoids lack a primary structure related to an amino acid sequence, there are direct analogies that can be made between proteoids and proteins at higher structural levels. Folded proteins have secondary structures, which are sub-structures including \(\alpha\)-helices and \(\beta\)-sheets often associated with hydrogen bonding, and tertiary structures, which organize the secondary structures, yielding particular overall protein shapes. By analogy, shape-designed proteoids can have identifiable secondary structures, such as heads (e.g. red circular portions in Figure 3.1c) and tails (e.g. blue annular sector portions in Figure 3.1c), and the tertiary structures are related to the designed sizes, shapes, and relative placements of these secondary structures. Consequently, both secondary and tertiary structures of proteoids can be precisely controlled and directly mutated using human design and top-down lithography. By contrast, for proteins, genetic mutations are translated into different primary protein structures, which after folding, can give rise to different secondary and tertiary protein structures.

Beyond secondary and tertiary structures, two or more proteins can assemble into quaternary structures; the most common of these is the dimer. Similarly, through slow crowding, certain proteoid shapes can self-assemble into monodisperse quaternary structures, such as the chiral D, dimer, via shape-designed CPS. While this is already remarkable, beyond this, such chiral dimers can, in-turn, self-assemble to an additional level of complexity to form dimer microcrystals, such as HI-DX1. Thus, we have experimentally demonstrated that hard Brownian proteoids, when crowded, can self-assemble up to the quinary structural level of
proteins\textsuperscript{17}, which includes complex multi-protein structures such as crystals of protein dimers as well as viral capsid shells.

### 3.5 Conclusions

Although higher levels of structural organization of proteins are often associated with various types of attractive interactions between them, we have demonstrated experimentally that quaternary and quinary levels of structural organization of self-assembled proteoids can be realized without hydrogen bonding, hydrophobic interactions, or site-specific attractions between certain residues. Thus, enantiopure Brownian 2D systems of lithographic proteoids provide a highly controlled model system for studying hierarchical self-assembly in which only core shape, local entropy maximization, and history of crowding control the self-assembled structures. A random fluctuating force field, such as that caused by entropic Brownian excitations, and a weak applied external field, such as gravity, which causes slow crowding, together are sufficient to produce dimer crystallization of certain colloidal core shapes that have have been designed to promote CPS in the absence of in-plane attractive interactions. Although a variety of interactions no doubt contribute to certain protein structures, it is nevertheless evident that these are not required to achieve a dramatic level of hierarchically self-organized complexity. This suggests that crystals of certain dimerized proteins, or even more complex constructs of multiple proteins and other biomolecular objects, can potentially be made by a mechanism of intentionally reducing or suppressing attractive interactions, for instance by increasing charge-screening through manipulation of pH and ionic strength, and simply osmotically concentrating these proteins very slowly, using known methods of dialysis or evaporation.

We anticipate that our observations of hierarchical self-organization of shape-controlled proteoids will spark a number of exciting future directions. Experimentally, it would be
interesting to see if refining the designs of the shapes of proteoids could lead to suppression of the DX2 crystallization pathway, thereby enhancing the yield and size of DX1 microcrystallites. Although our 2D studies have revealed important mechanisms that lead to dimer crystallization, examining dimer assembly and crystallization of proteoids in 3D could potentially lead to more complex crystal phases that match those of 3D protein crystals. In addition, regarding the concept of dimers as compressible “quasi-particles”, it would be interesting to explore this further theoretically in the context of hydrodynamic theory. Examining crystallization of heterodimers made of two differently shaped proteoids could mimic protein dimer crystallization facilitated by chaperone proteins. Theoretically and numerically, it would be useful to establish predictions of the effective monomer diffusion rates in dense systems of hard Brownian proteoids through the identified TTR mechanisms. This, in turn would facilitate predictions of the aging of the system as monomers unite and combine into dimers, even in a crowded environment.
Appendix A. Supplementary information for Chapter 3

Supplementary Figures

Figure S3.1 HI-proteoid area fraction $\phi_A$, osmotic pressure $\Pi^{2D}$, and equilibrium constant $K$ depend the depth, $d$, measured from the top of the column. (a) Area fractions of monomers ($\phi_{AM}$, black circles) and of dimers ($\phi_{AD+}$, red squares). Solid lines are fits using eqs. S3.1 and S3.2, respectively; these lines intersect at $d^* = 101$ µm where $\phi_{AM} = \phi_{AD+}$. Dashed line: fit to an exponential rise for $d \leq 40$ µm, yielding the thermal-gravitational height of a monomer of $h_{gM} \approx 10$ µm. Bars indicate ± one standard deviation of measurements from the lines in different regions of $d$. (b) Total area fraction $\phi_A$ of proteoids (black circles; left axis) and two-dimensional osmotic pressure $\Pi^{2D}$ normalized by $k_B T/\lambda_p$ (blue squares; right axis) calculated by integrating $\phi_A(d)$. (c) Equilibrium constant $K$ of the dimerization reaction calculated using the law of mass action: $K = \phi_{AD+}/\phi_{AM}^2$, where by convention we choose $K = 1$ at $d = d^*$. Solid line: fit of measured $K(d)$ to $K = \exp \left[\left(\frac{d-d^*}{L_D}\right)\right]$, where $L_D = 9.0 \pm 0.2$ µm is a characteristic length scale associated with dimer formation.
Figure S3.2 Osmotic pressure and equilibrium constant $K$ of proteoids. (a) Measured two-dimensional osmotic equation of state of HI-proteoids, defined as the scaled 2D osmotic pressure $\Pi_{2D}/(k_B T/A_p)$ versus the area fraction $\phi_A$. The measurements are fit to $\Pi_{2D}(\phi_A)/(k_B T/A_p) = f\phi_A/[1-(\phi_A/\phi_{A,c})]$ where $f = 1.70 \pm 0.08$ is a dimensionless prefactor and $\phi_{A,c} = 0.458 \pm 0.006$ is a critical area fraction. (b) Natural logarithm of the equilibrium constant $K$ for dimerization as a function of $\Pi_{2D}/(k_B T/A_p)$; $K$ increases exponentially with applied osmotic pressure. Solid line: linear least squares fit, yielding a slope of $3.5 \pm 0.1$. 
Figure S3.3 Real-space structure and Fourier transforms of an isolated self-assembled chiral dimer crystallite (DX1) of chiral head-centered HC-proteoids. (a) Real space optical microscope image in which proteoids have been filled to a uniform black density and the space outside is uniformly white. Scale bar: 3 µm. The HC-DX1 lattice of dimers is rectangular; average lengths of primitive vectors (red arrows) are (µm): \( |a_1| = 5.1 \pm 0.1 \), \( |a_2| = 8.4 \pm 0.1 \); internal angle between \( a_1 \) and \( a_2 \) is \( \gamma = 90° \pm 2° \). (b) Fourier transform of the crystallite in (a): field amplitude in reciprocal space. Complex Fourier transform of (a): (c) Real part and (d) Imaginary part. Constructive and destructive interference effects are not the same in the real and imaginary parts. Certain peaks are visible in the real pattern (c) but not in the imaginary pattern (d), and vice-versa. Inverse complex Fourier transform of (c) and (d) yields the real space image in (a). White scale bars: 3 µm⁻¹.
Figure S3.4 Structure and Fourier transforms of an isolated self-assembled chiral dimer crystallite (DX2) of chiral head centered HC-proteoids. (a) Real space optical microscope image in which proteoids have been filled to a uniform black density and the space outside is uniformly white. Scale bar: 3 µm. The HC-DX2 lattice of dimers is oblique; average length of primitive vectors (red arrows) are (µm): |a₁| = 6.5 ± 0.1, |a₂| = 8.3 ± 0.2; internal angle between a₁ and a₂ is γ = 52° ± 2°. (b) Fourier transform of the crystallite in (a): field amplitude in reciprocal space. Complex Fourier transform of (a): (c) Real part and (d) Imaginary part. Constructive and destructive interference effects are not the same in the real and imaginary parts. Certain peaks are visible in the real pattern (c) but not in the imaginary pattern (d), and vice-versa. Inverse complex Fourier transform of (c) and (d) yields the real space image in (a). White scale bars: 3 µm⁻¹.
Supplementary Methods

Fabrication of Chiral Proteomimetic Colloids — Solid microscale particles composed of epoxy photoresist SU-8 (Microchem Inc.) are fabricated using top-down photolithography, yielding a stable aqueous dispersion of monodisperse polymeric prismatic platelets that have customizable shapes. To make proteomimetic colloids, or proteoids, we modify the shape of an achiral annular sector particle (ASP) by decorating the end of only one of its arms with a superimposed circular feature, thereby causing it to be identifiably chiral. Lithographic layout software (L-Edit, Tanner Research, Inc.) is used to design an array of a particular proteoid shape in a photolithographic negative mask, suitable for 5x reduction stepper lithography. We have designed three different types of chiral ASP shapes, which we refer to as head-interior (HI), head-centered (HC), and head-exterior (HE) proteoids, based on the position of the circular head relative to the annular sector, as shown in Figure 1c. We have considered pairs of each proteoid shape at various relative positions and orientation angles, and all types sterically inhibit one chiral pathway of in-plane dimerization, so that only D+ dimers can form. As designed on the quartz-chrome photomask, HI-, HC-, and HE-proteoids have inner radii of 8.9, 9.0, and 8.2 µm, outer radii of 13.0, 13.3, and 12.3 µm, head radii of 4.5, 6.7, and 6.7 µm, and opening angles of $\psi = 72^\circ$, 86°, and 88°, respectively. Here, the opening angle $\psi$ is defined as the interior angle between two radial lines that emanate from the mutual center of both circular arcs of the ASP. The first radial line simply intersects with the end of the arm of the ASP that does not have the head. The second radial line just barely touches the edge of the head region on the other arm of the ASP.

We have designed a single photomask that contains several different rectangular zones; each zone has a square array of transparent shapes of only one type of proteoid. For HI-, HC-,
and HE-proteoids, these zones have arrays containing 1931 x 555, 1229 x 521, and 1160 x 542 replicates, respectively; each proteoid is separated with neighboring proteoids by 10 µm. The photomask is made using an e-beam lithography system (MEBES, 50 nm feature size) loaded with a 6 in. x 6 in. x 0.25 in. quartz-chrome mask; after e-beam exposure, regions of the chrome layer are stripped away, leaving an array of transparent shapes of chiral ASPs. Dispersions of each type of proteoid are made by selectively printing corresponding regions of the photomask using an ultraviolet optical lithographic stepper (ASML, PAS 5500, i-line 365 nm ultraviolet (UV) light) that provides a 5x size reduction of features on the photomask. Thus, the spatial dimensions on the quartz-chrome photomask are 5x larger than the actual desired dimensions of the printed proteoids made of crosslinked SU-8.

To fabricate stable aqueous dispersions containing many millions of copies of identical chiral proteoids of a single head type, we spin coat a lift-off-release material, LOR-1A (Microchem Inc.), on a 4-inch diameter silicon wafer as a sacrificial layer, yielding a solid layer thickness of 120 nm after baking at a temperature of 200 ºC for 120 s and then cooling to ambient temperature of 25 ºC. Subsequently, a layer of SU-8-2001 negative photoresist is spin-coated on top of the LOR-1A layer, and the wafer is baked at 95 ºC for 90 s, yielding a layer of SU-8 that is ≈ 1 µm thick. Similarly prepared wafers are loaded into the stepper, which exposes the photoresist layer at 5:1 reduction with patterned UV light (dose = 200 mJ/cm²). After a post-exposure bake at 95 ºC for 60 s, the exposed SU-8 is crosslinked within the photoresist layer. Only one zone of the photomask, corresponding to an array of only one particular chiral proteoid shape, is used by the stepper for this exposure over the surface of a single wafer. After repeated exposures that cover ≈ 90% of the surface of the wafer with the array pattern (avoiding edges of the wafer), post-baking, and cooling to room temperature, the exposed wafer is immersed in a
SU-8 developer solution (1-methoxy-2-propyl acetate), which dissolves the unexposed and uncrosslinked SU-8, leaving an array of proteoids attached to the sacrificial LOR layer. After rinsing with isopropyl alcohol to remove any residual SU-8 and drying with a nitrogen gas stream, the printed SU-8 proteoids are released from the wafer by dissolving the LOR layer in a basic aqueous surfactant solution containing 40 mM sodium dodecyl sulfate (SDS, MP ultrapure) and 1.8% w/v tetramethylammonium hydroxide (TMAH, Sigma-Aldrich, 25% in water). Adsorbed SDS on the surfaces of the particles inhibits particle aggregation and enables uniform dispersion in the solution. We concentrate and wash the aqueous dispersion of proteoids by gentle and brief centrifugation (2500 rpm, 20 min), remove the supernatant containing residual dissolved LOR and TMAH, and then dilute the particles with an aqueous solution having [SDS] = 5 mM. To further reduce the concentrations of LOR and TMAH, we repeat this washing procedure 3 more times, yielding a stable aqueous dispersion of ≈200 million monodisperse proteoids produced from a single wafer in a 5 mM SDS solution.

Characterization of Chiral Proteomimetic Colloids — We characterize the printed SU-8 proteoids as shown in Figure 1d using a scanning electron microscope (JEOL, JSM-6700 FE-SEM, 5 kV). Top-down views of HI-, HC-, and HE- proteoids reveal highly uniform dimensions (averaged over at least 10 particles): outer radii, \( R_o = 2.48 \pm 0.04 \mu m, 2.51 \pm 0.02 \mu m, \) and \( 2.33 \pm 0.02 \mu m; \) inner radii, \( R_i = 1.72 \pm 0.02 \mu m, 1.78 \pm 0.02 \mu m, \) and \( 1.66 \pm 0.03 \mu m; \) head radii, \( R_h = 0.82 \pm 0.03 \mu m, 1.19 \pm 0.01 \mu m, \) and \( 1.16 \pm 0.02 \mu m; \) area of a face: \( A_p = 8.61 \pm 0.33 \mu m^2, 10.05 \pm 0.42 \mu m^2, \) and \( 9.35 \pm 0.65 \mu m^2; \) opening angle, \( \psi = 78 \pm 2^\circ, 99 \pm 2^\circ, \) and \( 101 \pm 2^\circ, \) respectively. As a consequence of lithographic exposure and development, these measured dimensions of SU-8 particles are about 5%-10% smaller than a prediction based on perfect 5x reduction of the designed dimensions; for the same reason, the actual opening angles of SU8
proteoids are larger than designed by about 10°-13°. Taking SEM edge views of released and redepoted proteoids, we find that the average thickness of proteoids is 1.0 ± 0.1 µm. Sharp corners in the design are slightly rounded, as a consequence of the optical diffraction of UV light during exposure; the radius of curvature associated with this corner rounding is ≈ 200 nm, approximately half of the wavelength of the UV light.

**Concentrating and Crystallizing Proteomimetic Colloids** — We prepare and slowly concentrate a 2D Brownian monolayer of proteoids as follows. To a dilute dispersion of proteoids, we add a dispersion of a nanoscale depletion agent (Invitrogen, 42 nm diameter polystyrene spheres, carboxylate stabilized, surfactant free), yielding a post-mixing proteoid volume fraction of ϕ ≈ 0.03% and a post-mixing depletion-agent volume fraction of ϕ_d = 0.5%. This depletion agent induces a facially anisotropic roughness-controlled depletion attraction (FA-RCDA). After a depletion agent is introduced, as a consequence of fabrication, each lithographic colloid has one face that is more strongly attracted to a flat smooth substrate with an energy significantly greater than thermal energy, k_BT, and an opposite face that is only weakly attracted to the substrate with an energy less than k_BT, where T is the temperature. This mixed dispersion containing ~10^5 proteoids is loaded into a rectangular optical cuvette (30 mm x 2.0 mm x 0.1 mm). The loaded cuvette is placed flat in the center of a standard glass microscope slide, and both open ends are sealed with UV optical glue (Norland, type 81); this glue also rigidly mounts the sealed cuvette to the slide without obscuring the ends of the optical cell. The cuvette is oriented horizontally, so that the earth's gravitational acceleration is perpendicular to the largest 30 mm x 2.0 mm area, corresponding to its lower and upper walls. The FA-RCDA causes a preferential attraction of only one face of each chiral ASP towards the lower flat smooth wall of the optical cuvette. After ≈ 10 hours of sedimentation while horizontal, a dilute enantiopure
monolayer of mobile proteoids has formed just above the lower cuvette wall; proteoids are observed to diffuse translationally and rotationally in this dilute monolayer. Screened electrostatic repulsions, arising from the adsorbed SDS, prevent the proteoids from irreversibly adhering to the lower wall and preserve lubrication between the proteoids and the lower wall even in the presence of the depletion attraction. The diameter and post-mixing $\phi_d$ of the depletion agent have been optimized to induce FA-RCDA that lead to > 99.9% enantiopure monolayer formation of proteoids, as determined by examining more than 1,000 proteoids that diffuse above the flat surface in the presence of FA-RCDA.

In order to slowly concentrate the particles and enable crystal formation without substantial non-equilibrium jamming of particles that can lead to disorder, we tilt the cuvette along its long axis at a small angle of 5° and monitor the slow sedimentation towards the lower end of the cuvette. After a period of three months, during which the particles become gradually crowded towards this lower end, we then image the column from the top to the bottom using an inverted microscope (Nikon TE2000) equipped with a 40× extra-long working distance objective lens (Nikon Plan Fluor ELWD, 0.6 numerical aperture) and a Nikon D5000 camera (4288 pixels x 2848 pixels). To obtain an expanded, panoramic view that extends deep into the column while maintaining fidelity of single-particle features, we digitally combine several images that overlap, identify monomers, dimers, and crystallites, and color the particles as shown in Fig. 2b using Photoshop. In addition, using a computer-automated camera control system, we take time-lapse images of a fixed region in the dense region of a monolayer at a rate of one frame every 3 minutes over about one month using the same type of microscope that has been tilted by 5°. We combine these images into time-lapse movies that reveal particle-scale reaction events in the
crowded system of proteoids. To enhance the clarity of individual proteoids in these movies, deblurring filtering has been employed in Photoshop.

**Determining Area Fractions of Proteoids** — To measure the area fraction of chiral proteoids as a function of depth, $d$, into the tilted 2D monolayer, bright regions inside the dark edges of proteoids in the combined micrograph are colored in green if they are in monomer (M) configurations and in red if they are in positive chiral dimer $D^+$ configurations using light-dark edge detection in Photoshop. The area fractions of monomers, $\phi_{A,M}$, and the area fraction of $D^+$ dimers, $\phi_{A,D^+}$, are measured by counting the different colored pixels in a thin rectangular strip, which has a height of 20 $\mu$m (i.e. along $d$) and a perpendicular width of 118 $\mu$m (i.e. the width of the entire image). This 20 $\mu$m strip is moved 10 $\mu$m successively downward and the number of pixels in each strip is counted. To overcome limitations created by diffraction in the optical images, we more precisely determine the area particle fractions, $\phi_A$, we convert the number of colored pixels to a bare particle area using a calibration based on SEM images. The average facial area per proteoid for each type of proteoid is obtained from SEM images (e.g. see Fig. 1d). For a given proteoid shape and fixed illumination conditions, we use Photoshop's edge-detection to select and count the number of colored light pixels inside its dark edge-boundary in the optical micrograph. We determine an average number of colored pixels per proteoid from least 15 proteoids. Since this number of colored pixels per proteoid is proportional to the area of a proteoid, we count the number of colored pixels in a 20 $\mu$m strip of the combined image and convert this number to a bare area by multiplying it with the SEM-average area per proteoid and dividing it by the average number of colored pixels per proteoid. This yields the average bare
area of proteoids within the strip, and we divide this area by the entire area of the strip to determine the bare area fraction \( \phi_A \) of proteoids in the strip.

We examine the behavior of the monomer and dimer area fractions as a function of depth in detail for HI-proteoids, shown in Fig. S3.1a. As a consequence of the applied gravitational 2D osmotic pressure, \( \Pi_{2D} \), the area fraction of monomers, \( \phi_{A,M} \), increases with depth \( d \) into the column. In the dilute gas-like region, we fit \( \phi_{A,M}(d) \) for \( d \leq 40 \mu m \) to a simple exponential increase expected for a barometric column, \( \phi_{A,M}(d) \sim \exp(d/h_{g,M}) \), yielding a characteristic thermal-gravitational height of monomers of \( h_{g,M} \approx 10 \mu m \). Deeper into the column, \( \phi_{A,M} \) reaches a maximum value of \( \approx 0.24 \) at \( d \approx 80 \mu m \). Beyond this peak, \( \phi_{A,M}(d) \) decreases rapidly, as the vast majority of monomers react to form dimers in a monomer-dimer reaction zone (80 \( \mu m < d < 120 \mu m \)). In the bottom of the column, \( \phi_{A,M}(d) \) fluctuates around 0.04; this non-zero value is likely to be a simple consequence of statistical sampling and the discrete nature of the particles (i.e. finding even versus odd numbers of particles in any given limited sampling area). Monomers in the dense region do not rapidly combine to form dimers because their diffusion around intervening dimers is very slow; tautomerization reactions do speed up the effective diffusion rate of monomers, but even so, the effective monomer diffusion rate that leads to dimer formation is still very low.

We perform a least-squares fit to the measured \( \phi_{A,M}(d) \) of HI-proteoids using a piecewise empirical equation that captures the peak as well as the residual area fraction of monomers, \( \phi_{A,R} \), deep within the column:

\[
\phi_{A,M}(d) = \begin{cases} 
\phi_{A,M}^* \left[ 1 + \exp\left(-\frac{(d-d_{0,M})}{h_{g,M}}\right) \right]^{-1} \left[ 1 + \exp\left(\frac{(d-d_{0,M})}{L}\right) \right]^{-1}, & d < d_{0,M} \\
(\phi_{A,M}^* - 4\phi_{A,R}) \left[ 1 + \exp\left(-\frac{(d-d_{0,M})}{h_{g,M}}\right) \right]^{-1} \left[ 1 + \exp\left(\frac{(d-d_{0,M})}{L}\right) \right]^{-1} + \phi_{A,R}, & d \geq d_{0,M}
\end{cases}
\]  
(S3.1)
where $\phi_{A,M}^*$ is a characteristic area fraction related to the amplitude of the monomer peak, $d_{0,M}$ is a reference depth near the peak, $h_{g,M}$ is a thermal-gravitational height of monomers, and $L$ is a characteristic length scale associated with the decrease in the monomer peak in the monomer-dimer reaction zone. This fitting formula resembles one that has been used for achiral ASP particles, except that here we have explicitly included $\phi_{A,R}$ in a continuous piecewise equation. The decrease of $\phi_{A,M}$ towards larger $d$ is represented by the second inverse factor in both pieces of the equation. When fitting, we fix $h_{g,M} \approx 10 \mu m$ obtained most accurately from the fit to the leading edge of the peak, and allow $\phi_{A,M}^*, d_{0,M}, L$, and $\phi_{A,R}$ to vary, resulting in a good agreement (correlation coefficient = 0.97) and fit parameter values of $\phi_{A,M}^* = 0.83 \pm 0.04, d_{0,M} = 53 \pm 2 \mu m$, $L = 29 \pm 3 \mu m$, and $\phi_{A,R} = 0.036 \pm 0.003$. Standard deviations of the measured points from the fitted line in three different regions ($60 \mu m < d < 90 \mu m$, $120 \mu m < d < 190 \mu m$, and $260 \mu m < d < 390 \mu m$) are shown as black error bars (i.e. total length of each bar represents ± one standard deviation) in Fig. S3.1a.

From the dilute gas-like region and into the reaction zone towards larger $d$, the area fraction of enantiopure chiral dimers (D+), $\phi_{A,D+}(d)$, increases rapidly and then slowly continues to rise in the dense dimer region. We modify a prior semi-empirical model for the depth dependence of the dimer area fraction of achiral dimers by now including a linear increase of $\phi_{A,D+}(d)$ in the dense region. In the present system of enantiopure chiral dimers, there is a larger, non-negligible compressibility at higher depths and applied 2D osmotic pressures. This has its origin in at least two sources: there is overall a higher density of monomers in the dense region as a consequence of suppression of one reaction pathway, and also dimer crystallites are forming in the dense region, and since these dimer crystals represent a higher packing efficiency, this also
contributes to local spatial density fluctuations in the system. We therefore model the
compressibility at high particle densities by including a linear component in a piecewise function.
Because dimer crystals have a higher area fractions that defected or disordered regions, large
dimer crystals can be detected by locating the small peak-like fluctuations of $\phi_{A,D+}(d)$ shown at
large $d$ in Fig. S1a. We fit the measured $\phi_{A,D+}(d)$ to:

$$\phi_{A,D+}(d) = \begin{cases} 
\phi_{A,D+}^* \left\{ 1 + \exp\left[-(d - d_{0,D+})/h_{g,D+}\right]\right\}^{-1} & \text{d < } d_{0,D+} \\
\phi_{A,D+}^* \left\{ 1 + \exp\left[-(d - d_{0,D+})/h_{g,D+}\right]\right\}^{-1} + \phi_{A,D+,L1}(d - d_{0,D+})/d_{0,D+} & \text{d } \geq d_{0,D+}
\end{cases}$$  (S3.2)

where $\phi_{A,D+}^*$ is a characteristic dimer area fraction associated with the degree of dimer formation
in the reaction zone, $d_{0,D+}$ is a characteristic reference depth associated with the initial rise in
$\phi_{A,D+}(d)$, $h_{g,D+}$ is a length scale associated with this rise, and $\phi_{A,D+,L1}$ is a slope describing the linear
increase of $\phi_{A,D+}(d)$ in the dense region. The fit agrees well with the measurements (correlation
coefficient $= 0.996$), yielding parameter values of $\phi_{A,D+} = 0.34 \pm 0.01$, $d_{0,D+} = 104 \pm 1 \, \mu m$, $h_{g,D+} =
14 \pm 1 \, \mu m$, and $\phi_{A,D+,L1} = 0.024 \pm 0.004$. The red bars in Fig. S3.1a represent ±1 standard
deviation of departures of the measured points from the fits in the same regions of $d$ previously
defined for the monomer area fraction. In the dense region, the larger bars for $\phi_{A,D+}$ as compared
to $\phi_{A,M}$ are a consequence of the additional effect of dimer crystal formation. Summing $\phi_{A,M}(d)$
and $\phi_{A,D+}(d)$ gives the total area fraction $\phi_A(d)$ of proteoids, as shown in Fig. S3.1b (left axis).

Gravitational 2D Osmotic Pressure —— Using $\phi_a(d)$ for the system of HI-proteoids
shown in Fig. S3.1b (left axis), we calculate the gravitational 2D osmotic pressure $\pi_{2D}$ by
summing up the effective buoyant gravitational forces of all proteoids above a given height $z$:

$$\pi_{2D} = (k_B T / A_p) \int_z^{\infty} \phi_A(z')(dz'/h_{g,M}) \ , \text{ where } T \text{ represents temperature, } A_p \text{ is the facial area per}$$
proteoid, $h_{gM} = 10 \, \mu m$ is the thermal-gravitational height of a monomer proteoid, and $z' = 390 \, \mu m - d$ is a variable of integration that points upward from the bottom of the column. We present the results of this numerical integration as the scaled 2D osmotic pressure, $\Pi_{2D}/(k_B T/A_p)$, shown in Fig. S3.1b (right axis).

**Equilibrium Constant of the Monomer-Chiral Dimer Reaction** — For chiral proteoids that have shapes that sterically suppress one chiral dimerization reaction pathway, the equilibrium reaction can be written as $2M \rightleftharpoons D^+$. Using the law of mass action, we calculate the equilibrium constant $K = \phi_{A,D^+}/\phi_{A,M}^2$ of this dimerization reaction in the reaction zone, where both $\phi_{A,M}$ and $\phi_{A,D^+}$ vary systematically, as shown in Fig. S3.1c for HI-proteoids. We observe that $K$ increases exponentially with $d$ in this region. Here, we have set $K = 1$ where the area fraction of monomers equals the area fraction of dimers at $d^* \approx 101 \, \mu m$. We fit $K(d)$ to an exponential equation: $K = \exp[(d-d^*)/L_D]$, where $L_D$ is the characteristic length scale associated with the rise in $K$ and dimer formation in the reaction zone. This fit describes the measured $K(d)$ well and yields $L_D = 9.0 \pm 0.2 \, \mu m$.

**2D Osmotic Equation of State** — The system’s 2D osmotic equation of state is determined by a scaled osmotic pressure as a function of area fraction $\phi_A$, as shown in Fig. S3.2a. For $\phi_A < 0.39$, the equation of state $\Pi_{2D}(\phi_A)$ can be fit accurately to a semiempirical equation $f\phi_A/[1-(\phi_A/\phi_{A,c})]$, where $f$ is a dimensionless prefactor and $\phi_{A,c}$ is a critical area fraction, yielding the parameter values of $f = 1.70 \pm 0.08$ and $\phi_{A,c} = 0.458 \pm 0.006$. This equation captures ideal gas behavior at low $\phi_A$ and a non-linear divergence towards a critical area fraction $\phi_{A,c}$ associated with packing or jamming. Since the functions $\Pi_{2D}(d)$ and $K(d)$ are both known, we can simply
plot $\ln K$ versus the scaled $\Pi_{2D}$ for $d$ in the reaction zone, as shown in Fig. S3.2b. The measured dependence is linear, so the equilibrium constant of the dimerization reaction increases exponentially with the applied 2D osmotic pressure. A linear least squares fit in Fig. S3.2b agrees well with the measurements, yielding a slope of $3.5 \pm 0.1$. Thus, the dimerization reaction of the Brownian proteoids is effectively driven by the applied 2D osmotic pressure in the gravitational column. This dependence includes the significant effective compressibility of lock-and-key dimer configurations.

**Complex Fourier Transforms** — 2D complex Fast Fourier Transforms (c-FFTs) are calculated using Matlab after image processing using Photoshop. Measured microscope images in 24-bit RGB color are converted to unsigned 8-bit grayscale images. To enhance contrast in an image, the interiors of proteoids in a single dimer crystal are selected and colored black in Photoshop; after inverting this selection, regions outside the dimer crystal are colored white. This high-contrast image of a single crystallite is resized and centered so that it fits well within a white region of $2048 \times 2048$ pixels without clipping. In Matlab, this image is converted to double-precision numerical data. Using Matlab's built-in function (fft2), we calculate the 2D c-FFT of crystal image and shift the low frequency component to the center of spectrum for visualizing (fftshift). We display the real part and also the imaginary part of the 2D c-FFT in reciprocal space; values of the complex field are double precision numbers. At the centers of these real and imaginary parts of the c-FFTs, which are effectively scattering patterns, the field amplitudes are typically a few orders of magnitude higher than peaks at higher wavenumbers that reflect the self-assembled dimer crystal structures, so we mask a small region of central pixels. In a given c-FFT, non-central peaks in either the real part or the imaginary part of the c-FFT contain both large positive numbers and also large negative numbers, so we color-code the amplitudes to
be black for zero field and brighter versions of two different colors for positive and negative field amplitudes. We choose to emphasize and report real and imaginary parts of the complex field in this manner, rather than the equivalent information contained in the field magnitude (i.e. amplitude) and phase, because interference effects are more directly evident in the different patterns of Bragg peaks, as shown in Fig. 3.3 and Figs. S3.3 and S3.4. We have verified that the high-contrast image of a dimer crystallite can be reconstructed by performing an inverse c-FFT using the reported real and imaginary parts obtained by c-FFT.

**Supplementary Discussion**

We conjecture that HI-proteoids have formed the largest dimer crystallites under the same conditions for the following reasons. A head-interior feature does not interfere with rotations of individual monomer proteoids as much as head-centered and head-exterior features. Thus, the rates of dimerization and tautomerization reactions will be correspondingly higher, since these reactions depend on the Brownian sampling of relative orientations of nearest neighboring proteoids. This Brownian sampling is related to the effective rotational diffusion coefficients of monomer proteoids in the crowded environment; protrusions that extend outside of the outer circular arc of the ASP sub-structure, which is the case for HC- and HE-proteoids, would tend to lower such effective rotational diffusion coefficients. Likewise, once dimers have been formed, the rate of crystal growth is also related to the rotations of proteoid dimers near the surface of the growing crystal, since these proteoids must be properly oriented in order to be incorporated into the crystal. Less compact dimer shapes that have protrusions do not rotate as readily as more compact dimer shapes that do not have protrusions, so again the kinetics of the growth of dimer crystals would be faster for HI-proteoids than for HC- and HE-proteoids.
Beyond analogies to proteins, the structures of the proteoids that we have fabricated and studied are also reminiscent of lyotropic lamellar phases of surfactant-like or lipid-like molecules that have heads and tails. For instance, the layered dimer structures of the DX1 and DX2 dimer crystals resemble to a certain extent interdigitated bilayer structures that can form of molecules that have hydrophilic head groups and hydrophobic tail groups. For both DX1 and DX2, the circular heads are positioned proximate to the heads of neighboring proteoids, and the ASP-tails are also proximate and interdigitating. These alternating regions of high densities of heads and tails lead to very strong spatial correlations of proteoid sub-structures, and this can also be seen in self-assembled lamellar phases. However, there are some differences, too, since the dimer crystals of proteoids have ordered arrangements of heads and tails within and between layers, whereas surfactant and lipid systems often have disordered liquid-like structures within individual layers.

Although we have used optical microscopy to examine microscale Brownian proteoids made using a stepper that has a minimum feature size specification of ≈ 300 nm, our methods can be directly extended to smaller proteoids using existing high-end lithographic steppers that offer minimum features sizes down to ≈ 30 nm. For such smaller proteoids, superresolution optical imaging, in-situ "wet" transmission electron microscopy, or standard TEM on fixed samples could be used instead of optical microscopy. Such smaller proteoids would potentially enable faster rates of crowding and crystallization, even if the underlying roles of core geometry and entropy maximization are the same.

The self-assembled quinary structures of hard proteoids that we have observed can be subsequently fixed to the substrate and retained by introducing a strong attractive colloidal interaction. This can be achieved in a variety of ways, such as by adding a depletion agent that
has a larger size, by introducing divalent ions of the opposite sign compared to the charges on the surfaces of the proteoids (e.g. by dialyzing with a solution of MgCl$_2$ or CaCl$_2$, yielding Mg$^{2+}$ or Ca$^{2+}$), and/or by modifying the pH of the aqueous continuous phase to protonate surface charges on the proteoids.

**Future Directions**

Exploring different histories of osmotic compression, such as varying the tilt angle in a time-dependent periodic manner, may enhance annealing, thereby reducing defects and promoting the growth of larger dimer crystallites. Likewise, turning on in-plane attractions, for instance by adjusting the concentration of a second depletion agent having a larger size, could affect the resulting sizes and structures of dimer crystallites. Moreover, developing methods of fixing 2D dimer crystallites to the substrate (e.g. by dialyzing the aqueous phase with a salt solution that would sufficiently reduce the barrier provided by the Debye screened repulsion) or transferring them to other interfaces would be useful. Just as we have designed shapes of the proteoids to suppress the D- dimerization pathway and select only the other D+ pathway, we also anticipate that it may be possible to suppress the formation of one of the two competing crystal phases and selectively form only a single crystal type by further refining the shapes of the monomer proteoids. Furthermore, manipulating the process of crystallization and self-assembled crystallites by applying external electromagnetic fields could yield field-controllable structural transitions. Furthermore, predicting the distribution of sizes and types of dimer crystallites as a function of time in a slowly compressed system of proteomimetic shapes would also be useful.
References


Chapter 4 – A Fluctuating Brownian Quasi-Crystal of Pre-Assembled Colloidal Penrose Tiles

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4.1 Introduction

Optical projection stepper nanolithography has been developed to such a remarkable degree that electronic circuit elements having ≈10 nm features are now mass-produced over large areas at high throughput with amazingly low defect rates\textsuperscript{1-2}. Modern central and graphics processing units are made using optical nanolithography in extremely clean, highly temperature-regulated, and nearly vibration-free fabrication environments\textsuperscript{2}. Such processing units typically have extraordinarily complex multi-layer nano-architectures composed of a number of materials, representing an amazing achievement of functional materials that possess an extraordinary information density and structured connectivity that is beginning to rival that found in highly evolved biological systems. Thus, optical stepper lithography currently provides a method of
large-area patterning with nanoscale features that is both commercially and also scientifically useful. While stepper lithography is primarily a two-dimensional (2D) non-contact patterning method, when it is combined with development, stripping, and deposition processes, particularly electron beam deposition of metals and chemical vapor deposition of dielectric materials, and nanopositioning and alignment systems, multiple exposures using different mask patterns can be used to create quasi-three-dimensional nanocircuitry containing more than thirty layers in some cases.

By contrast, the bottom-up approach self-assembly of colloidal particles has only been partially successful in building very complex structured and functional materials out of nanoscale or microscale particulate components. While bottom-up approaches offers the potential advantage of parallel assembly of desired large-scale structures in three dimensions (3D)\(^3\), they are often limited by high structural defect rates that are unacceptable in many applications, so large-scale precision nanocircuitry offering desirable features, such as addressability at low defect rates, has not been achieved by this method. While active biologically directed growth can produce highly organized living structures that are genetically encoded, such hierarchically organized superstructures have not been produced by non-genetic accessible forms of directed assembly so far.

A beautiful example of a hierarchically organized static superstructure is Penrose's P2 kite-dart tiling, which exhibits pentagonal quasi-crystalline (QC) order\(^5\)\(^-\)\(^7\). In the P2 tiling, individual kites and darts are organized locally into motifs, such as pentagonal stars, and these motifs in turn are organized into a variety of superstructural patterns that become increasingly complex at larger length scales\(^8\). Because routes for self-assembling complex colloidal building blocks into low-defect multi-scale dynamic superstructures remain limited, alternative methods
other than self-assembly are being explored. Although local motifs, which are consistent with QC order, have been assembled by manipulating spherical colloidal particles using holographic laser tweezers, no fluctuating Brownian system that exhibits the degree of hierarchical complexity inherent in Penrose's P2 tiling over a large area has yet been experimentally created and studied. Thus, a general method of creating multi-scale hierarchically organized systems of Brownian colloidal particles that have desired shapes at high densities is needed. Here we show that such systems of mobile particles can be created in 2D through a highly parallel lithographic printing and release into a solution-dispersion containing a depletion agent. This simultaneous multi-particle fabrication, pre-assembly, and release approach, which we call Litho-PAMs, enables us to control the sizes, shapes, and initial positions and orientations of many colloidal building blocks, yielding complex multi-scale monolayers of mobile tiles at high densities that are beyond current self- or directed-assembly methods. Using Litho-PAMs, we have produced a randomly fluctuating Penrose P2 QC of mobile kite and dart tiles, and, through high resolution time-lapse optical microscopy, we study dynamics of these tiles, motifs, and superstructures. The five-fold Penrose quasi-crystal in a 2D Brownian system of darts and kites has long-range quasi-periodic order and then shows that after release and equilibration at a high sub-unity particle area fraction, entropic fluctuations cause a power-law decay in the spatial pair correlation function. This is reminiscent of Berezinskii-Kosterlitz-Thouless theory (BKT), which, when applied to systems of hard Brownian disks by Nelson-Halperin-Young (NHY), yielded a theory of the 2D hexatic phase. Simultaneously, local five-fold bond-orientational order is still preserved over long-range. Since the fluctuating Penrose system exhibits a combination of liquid crystalline as well as quasi-crystalline order, we call this new kind of material a liquid quasi-crystal (LQC). Scattering patterns, given by Fourier transforms of...
micrographs of the kite-dart Penrose LQC, reveal ten azimuthally smeared peaks, a direct consequence of the entropic fluctuations combining with the quasi-crystalline arrangement of the building blocks. Thus, the LQC phase represents an exotic phase of condensed matter that combines key features of both quasi-crystals as well as liquid crystals. The pentatic LQC phase has only local quasi-periodic order yet still possesses long-range five-fold bond-orientational order, even as the hard tiles fluctuate. Moreover, by removing a confining wall, we reveal the melting of this fluctuating quasi-crystal through a pentatic liquid quasi-crystalline (LQC$_5$) regime.

Generally, Litho-PAMs are dense fluctuating systems of shape-designed colloidal particles that can possess extremely high levels of multi-scale complexity, including but not limited to multi-tile QCs. We anticipate that our approach can be extended to create pre-assembled multi-scale fluctuating Brownian systems of designer shapes in 3D through holographic$^{25,26}$ or stereolithographic$^{27,28}$ writing methods. While still below the levels of structural and dynamic complexity inherent in biological organisms, our approach opens up the experimental creation and study of highly advanced forms of condensed matter that only simulations could previously access.

4.2 Materials and Methods

4.2.1 Mask Design of a Desired Complex Pre-Assembly of Custom-Shaped Tiles

Microscale kite-shaped and dart-shaped prismatic tiles are fabricated out of epoxy-based negative photoresist SU-8 (Microchem Inc.), and these tiles are spatially and orientationally organized prior to release using the following top-down photolithography process. A quartz-chrome lithographic mask of kite and dart tiles, based on the Penrose P2 kite-and-dart quasi-
crystalline pattern, is designed at a desired tile area fraction of $\phi_{A,\text{des}}$. Each kite is a convex quadrilateral having two adjacent sides of designed shorter length $C_{\text{des}}$ and two adjacent sides of designed longer length $D_{\text{des}}$, where a 144 deg interior angle is between the two shorter sides and the other interior angles are 72 deg (see Fig. 4.1a, scanning electron micrograph (SEM) inset). The ratio of two side lengths, $D_{\text{des}}/C_{\text{des}}$, is the golden ratio (i.e. 1.618..). Each dart is a concave quadrilateral having side lengths $C'_{\text{des}}$ and $D'_{\text{des}}$, which are nearly the same as $C_{\text{des}}$ and $D_{\text{des}}$ of the kites, and four interior angles: 216 deg (concave vertex), 72 deg (convex vertex opposite the concave vertex), and two 36 deg (convex vertices at the two sharp tips) (see Fig. 4.1c, SEM inset). A P2 quasi-crystalline pattern containing $\approx10^6$ kite-and-dart tiles is digitally designed using lithographic layout software (L-Edit, Tanner Research, Inc.). An idealized P2 pattern is generated with thin lines, and subsequently these lines are thickened to determine vertices of the tiles at $\phi_{A,\text{des}} \approx 0.78$. The edges of these kites and darts are then identified as residing just inside these thickened lines, and coordinates of their vertices are obtained. The shorter and longer side lengths of the kites are designed to be 31.3 $\mu$m and 50.7 $\mu$m, respectively, on the quartz-chrome mask; after 5x reduction stepper optical lithographic printing, these mask dimensions lead to ideal edge lengths $C \approx 6.2$ $\mu$m and $D \approx 10.1$ $\mu$m of the prismatic SU-8 kite particles. The shorter and longer side lengths of the darts are designed to be 29.4 $\mu$m and 47.6 $\mu$m, respectively, on the mask, leading to $C' \approx 5.8$ $\mu$m and $D' \approx 9.5$ $\mu$m of the prismatic SU-8 dart particles. The overall lateral dimensions of the SU-8 tiles have been chosen to ensure that diffraction during the UV printing process does not cause them to become irreversibly bonded together through unwanted crosslinking in the spaces between them when printed at high densities. The separation between the printed SU-8 tiles at $\phi_{A} \approx 0.78$ is uniformly $\approx1.2$ $\mu$m, which is about 4 times the minimum feature size that can be printed by the particular UV stepper which we have used. Thus, although
our pre-assembled pattern of kite and dart tiles is inspired by the classic P2 construction that involves infinitesimally thin lines, it is distinguishably different, since our new variation of this classic pattern of lines enables us to set $\phi_{\text{latex,des}}$ and determine the vertices of all constituent kite- and-dart tiles in a well-defined manner. In order to confine these mobile kite and dart particles and to maintain a fixed $\phi_i$ after release, we have designed large millimeter-scale pentagonal-shaped confining walls. Each confining wall has a lateral thickness of about $\approx 100 \ \mu m$ and an inner edge length of $\approx 2 \ mm$, making contact with the outermost confined Penrose tiles. This confining boundary is also made out of SU-8 photoresist and is printed together with the particles in the same exposure step.

4.2.2 Lithographic Production of a Desired Complex Pre-Assembly of Custom-Shaped Tiles

To observe the Penrose kite and dart particles before, during, and after release, we use a transparent glass wafer as a substrate, and we record in-situ time-lapse images using bright-field transmission microscopy. A clean glass wafer (4 inch diameter, 500 $\mu m$ thick, borosilicate) is pre-baked on a hot plate at 200 °C for 3 minutes to remove adsorbed moisture from its surface and is then cooled to 25 °C. Onto this wafer, we spin-coat a sacrificial release layer of Omnicoat (Microchem Inc.) using a spin-coater (Headway Research Inc., PWM32 Spinner, 500 rpm for 5 s with acceleration 100 rpm/s; 3000 rpm for 30 s at acceleration 300 rpm/s), yielding an optically transparent thin layer having a thickness of about 15 nm after baking at 200 °C for 1 minute and then cooling to 25 °C. Subsequently, we spin-coat a layer of SU-8-2002 (Microchem Inc.) negative photoresist on top of the Omnicoat layer and then bake at 95 °C for 90 s, yielding an optically transparent solid, yet uncrosslinked, layer of SU-8 that is $\approx 2 \ \mu m$ thick after cooling to
25 °C. This coated wafer is exposed to patterned UV light (365 nm, typical energy dose 180 mJ/cm²) through the designed photomask (Digidat Inc., 6 inch x 6 inch x .25 inch Chrome on Quartz) in a lithographic stepper (ASML, PAS 5500/200, 5x reduction, i-line). Our printing job routine on the stepper yields 14 evenly separated identical dies containing Penrose kite-and-dart SU-8 particles with a boundary distributed on the wafer with ≈18 mm between dies. This patterned UV light triggers crosslinking of the oligomeric epoxy SU-8 photoresist, and this wafer is post-exposure baked at 95 °C for 75 s to enhance the rate of cross-linking reactions. After cooling to 25 °C, this post-baked exposed wafer is submerged in organic SU-8 developer (1-methoxy-2-propyl acetate, Microchem Inc.) for 4 minutes to remove the uncrosslinked SU-8, rinsed with isopropyl alcohol to wash away any residual developer on the wafer, and dried using a nitrogen gas stream. The result is a set of prismatic kite and dart Penrose particles in a P2 pattern; these particles are bound to the Omnicoat release material on the wafer, yet they are entirely disconnected from each other, despite the high particle area fraction. To achieve this, we have adjusted the energy dose and post-bake duration for SU-8 as described above, optimizing this lithography process for transparent glass wafers. Transmission optical microscopy through the glass wafer in an inverted imaging configuration provides better quality images of the SU-8 particles, by comparison to reflection optical microscopy of the particles through a thicker layer of solution above an absorbing and reflecting silicon wafer limited range of actual printed yet. The actual printed $\phi_A$ of SU-8 Penrose tiles, having the same spatial P2 arrangement defined in the mask, can also be adjusted over a limited range near $\phi_A,des$ by tuning the exposure's energy dose through the stepper and also through adjustment of post-exposure baking conditions.
4.2.3 Releasing Pre-Assembled Custom-Shaped Tiles to Form a Fluctuating Brownian Monolayer

To obtain large areas of stable fluctuating particles in a monolayer, controlling their release is essential. If the release occurs too rapidly, then concentration gradients of the release material can drive discrete particles out of the monolayer, leading to undesirable vacancies and defects in the overall structure. The composition of the release solution is also important, and including a stabilizing agent, to prevent irreversible aggregation of the SU-8 tiles, as well as a depletion agent, to overcome vertical entropic excitations and keep tiles in the monolayer, is typically necessary. Thus, the pre-assembly method that we present here is very different than prior 2D self-assembly experiments in which monolayers of shape-designed lithographic colloids were randomly deposited at dilute area fractions in a monolayer and subsequently concentrated slowly using gravitational sedimentation in the presence of roughness-controlled depletion attractions\(^\text{29}\). This prior approach of concentrating particles actually creates a gradient in particle area fraction throughout a tilted optical cell; whereas, by contrast, the pre-assembly method that we present here provides a uniform area fraction over a very large surface area. Thus, pre-assembly avoids potential issues of out-of-equilibrium jamming during crowding as well as spatial gradients in $\phi_z$ that were inherent in this past methods of 2D self-assembly.

After development of the SU-8 photoresist, yet prior to release, around each die on the dry glass wafer, we build a solid square-frame enclosure of four elastomeric polydimethylsiloxane (PDMS) walls. The purpose of these PDMS walls is to confine a dissolving solution that is added later to release the SU-8 particles. We first make a solid disk-like layer of PDMS elastomer that is about 3 mm thick and 5.5 cm diameter in a small petri dish, then cut this into a square frame (inner edge length 1.3 cm, lateral wall thickness 0.2 cm),
remove this square frame from the petri dish, and attach it to the glass wafer surrounding the die of SU-8 particles. To make the disk-like layer of PDMS, we mix a PDMS elastomer and its curing agent (Sylgard 184, weight ratio 10:1 of elastomer:curing agent) on a clean petri dish, gradually de-gas the PDMS at 25 °C for 12 hr, and then place the petri dish in a vacuum oven at 80 °C for 2 hr to remove any remaining entrapped bubbles and to cure the PDMS. Prior to attaching the PDMS walls to the glass wafer, we have removed the Omnicoat layer outside of the die region using a cotton-tipped applicator soaked in release solution. These PDMS walls form a water-impermeable bond with the glass wafer around a given die, and the bonded PDMS square frame and glass wafer effectively form a chamber (i.e. well) into which a release solution-dispersion can be loaded.

To release the kite-and-dart particles slowly and yet preserve their pre-assembled organization in the monolayer without introducing strong in-plane attractions between them, we have formulated an aqueous release solution-dispersion that contains a basic release agent (tetramethylammonium hydroxide, TMAH, Sigma-Aldrich, 1% w/v) which dissolves Omnicoat, a stabilizing agent (sodium dodecyl sulfate, SDS, MP Biomedicals, ultrapure, 10 mM) which prevents irreversible aggregation of released SU-8 particles, and a depletion agent (carboxylate stabilized, surfactant free polystyrene spheres, Magsphere Inc., 52 nm diameter, 1% w/v solids) which strongly inhibits released SU-8 particles from being entropically excited normal to the surface of the glass and out of the monolayer. We fill the PDMS well completely with this release solution-dispersion, and we immediately place a cover slip over the filled PDMS to reduce evaporation, which can lead to undesirable convection. As SU-8 particles are released, anionic dodecyl sulfate ions adsorb onto their surfaces, thereby providing stabilizing screened-charge electrostatic repulsions and preventing irreversible aggregation of these particles when
they collide with each other in the monolayer. The depletion agent induces a roughness-controlled depletion attraction (RCDA) that causes a preferential attraction of a face of each SU-8 particle towards the flat smooth surface of the glass wafer, whereas in-plane attractions between rougher edges of particles are small compared to $k_B T$. We have optimized the concentrations of SDS, the depletion agent's diameter and volume fraction, the thickness of the SU-8 particles, and the type and thickness of sacrificial layer to keep all released kite-and-dart particles in the monolayer for an extended period of time (i.e. at least several days). Otherwise, at other conditions, some of the released SU-8 particles can be excited and leave the monolayer vertically creating unwanted defects. Excitations that can cause the SU-8 particles to leave the plane include entropic Brownian motion (i.e. thermal fluctuations) or by convective flows driven by concentration gradients of the release material as it dissolves. The PDMS walls have been designed to be tall enough (i.e. 3 mm high) to keep any residual convection near the cover slip away from the monolayer, suitable for time-lapse imaging over many days.

### 4.2.4 High Resolution Time-Lapse Particle Imaging by Optical Brightfield Transmission Microscopy

After filling and covering the PDMS well, in-situ images are taken before, during, and after particle release using an inverted brightfield microscope (Nikon Eclipse TE2000) equipped with a $20\times$ objective lens (NIKON CFI60 Plan Achromat, 0.4 numerical aperture), a $10\times$ objective lens (NIKON Plan Achromat, 0.25 numerical aperture), and a Nikon D5000 camera (4288 pixels x 2848 pixels, silent mode). The image contrast and resolution are sufficiently large that even for the lower power $10\times$ objective, each particle's position and orientation can be readily seen over the entire field of view. Individual images showing a fixed region of Penrose
tiles are taken at a rate of one frame every 60 s over using a computer-automated camera control system.

4.2.5 Fourier Transforms of Microscope Images Containing Filled Kite and Dart Tiles

Measured images in 24-bit RGB color are converted to unsigned 8-bit grayscale and then cropped into a square region of 2048 pixels x 2048 pixels. To enhance contrast in an image, the interiors of kite-and-dart particles are filled with black and regions outside the particles are filled with white in Photoshop. We use ImageJ to perform a 2D Fast Fourier Transform (FFT) of these black-and-white images, yielding intensity and phase. We display the FFT intensity using a color lookup table (C-LUT) to convert grayscale intensity values to colors. In some cases, we average several FFT intensities of images taken at different times together in order to create a time-averaged FFT.

4.2.6 Motif Superstructural Orientational Pair Correlation Function (MSO-PCF)

In structurally complex and hierarchically organized multi-scale materials, readily identifiable local groups of particles (i.e. local polymorphic configurations) can be defined as a distinguishable motif containing a prescribed set of one or more types of building blocks (e.g. particles) in specific relative arrangements, typically that are either densely arranged or even attractively bonded. Motifs can fluctuate and deform away from idealized symmetric configurations as a consequence of thermal fluctuations, but their fundamental relative local structures must be maintained over time in equilibrium and not break up in order for the motif to be a well-defined and enduring thermodynamic entity while the system is in equilibrium with a
heat bath at temperature $T$. Motifs can be defined purely through geometric assignment, irrespective of whether or not attractive bonds exist between the building blocks (i.e. individual particles). Thus, motifs can be defined for systems of particles that have hard interactions if they are at large enough densities that the motifs are maintained over time. The center of a particular motif can be identified (e.g. through a mass-weighted approach involving constituent building blocks), and certain motifs can possess an axial orientation or even a pointing direction, depending upon their specific geometries and broken symmetries. In certain systems, motifs can be organized into local superstructural sets, wherein local groups of motifs at different spatial locations within the material have an identifiably similar geometrical configuration. These local superstructural sets of motifs also are fluctuating under thermal excitations, leading to time-varying relative locations of the centers of motifs within a given local set, which can be characterized in terms of a set of time-varying superstructural angles corresponding to that particular local set of motifs.

In certain hierarchically organized materials, motifs are recurring features in a pattern, wherein one motif is surrounded by other similar motifs, such that a particular motif can be identified as a central motif. If this is the case, then a set of superstructural angles at any given instant in time can be defined between the center of the central motif and the centers of the other surrounding motifs. If a set of motifs is symmetrically arranged but lacks a central motif, then the center of the set can be defined by the average center positions of all motifs in the set.

We call the angles between the center of a local superstructural group of motifs and a particular exterior motif "superstructural angles", since these angles are defined with respect to the centers of motifs, rather than the centers of individual particles. Also, we refrain from using the terminology "bond orientation" since bonding as a consequence of attractive interactions is
not present in systems of hard particles. In other words, the set of superstructural angles of a
given local set of motifs is a purely geometrical property of their configuration, not of the degree
of enthalpic attractions between constituent building blocks, which is normally associated with
bonding. Thus, our definition of motif superstructural angles represents a higher order collective
geometrical property as compared to, for example, the traditional "bond orientations" of six
individual disks surrounding a central disk in a simple hexatic LC phase\textsuperscript{30-31}. Likewise, the
orientational correlations between pairs of sets of motifs, calculated using the superstructural
angles in a first set of motifs with respect to the superstructural angles in a second set of motifs,
can be examined as a function of the separation $r$ between these motifs to determine if
superstructural orientational order is maintained over distances larger than the minimum motif-
motif separation. This definition of a higher-order collective geometrical property based on local
superstructures of motifs is especially useful when local collective translational or rotational
motion of individual particles would potentially indicate orientational disorder, yet hide
hierarchical order at larger scales that may persist in equilibrium despite the mobility of some
individual particles.

To calculate the motif superstructural orientational pair correlation function, it is
necessary to define the motif, which can contain one or more different types of particles. Here,
for simplicity, we consider a motif that consists of $m$ particles of the same type (e.g. a pentagonal
star made up of $m = 5$ kites). The centers of motifs do not necessarily have to reside inside a
physical building-block particle. We define the center of a motif as the mass-weighted average
over all of its $m$ particles. Having defined the locations of motifs, we then define a
superstructural set of motifs as a local collection of $m$ motifs arranged within a certain local
range (e.g. a superstructural set of $n = 5$ pentagonal star motifs around a central pentagonal star
motif). The center of a superstructural set is defined as the mass-weighted average over all of its $n$ motifs in the set. Here, if there is a central motif in a superstructural set, we exclude this central motif from the value of $n$, so $n$ essentially refers to the number of external motifs in the set. By nature of the definitions of motifs and superstructural sets of motifs, we assume $m \geq 2$ and $n \geq 2$.

To calculate the motif superstructural orientational PCF (MSO-PCF), we define a first set of motif superstructural angles $\{\theta_{a,j}\}$ associated with a first superstructural set of $n$ motifs at an average first motif set position $r_a$, where $a$ is an integer label associated with the first superstructural set of motifs and $j$ is an integer index that ranges from 1 to $n$ motifs within that set. Likewise, we define a second set of $n$ motif superstructural angles $\{\theta_{b,k}\}$ associated with a second superstructural set of $n$ motifs centered at an average second motif set position $r_b$, where $b$ is an integer label associated with the second superstructural set of motifs and $k$ is an integer index that ranges from 1 to $n$ motifs within that set. All angles are defined relative to a direction in the fixed lab frame (e.g. positive $x$-direction), and each set of angles is defined as an ordered list in a counterclockwise direction from this direction. The spatial separation $r$ between the centers of the first and the second superstructural sets of motifs can thus be defined as $r = |r| = |r_b - r_a|$. Typically, the minimum accessible value of $r$ in this calculation will be significantly larger than the maximum spatial dimension of the constituent particles.

Having defined the superstructural angles at a particular instant in time, we then can calculate the $n$-fold MSO-PCF by adding up the complex exponentials of the angles multiplied by a symmetry number associated with the number of motifs in a superstructural set:

$$G_n^{\text{MSO}}(r) = G_n^{\text{MSO}}(|r_b - r_a|) = \left\langle \frac{1}{n} \sum_{l=1}^{n} \exp\left[i n (\theta_{b,l} - \theta_{a,l})\right] \right\rangle_{\forall a, \forall b > a},$$

(4.1)
where the condition \( b > a \) in the averaging avoids double-counting the same pair of superstructural sets of motifs. When superstructural sets of motifs possess the same orientation angles (e.g. as in a perfect superstructural crystal or quasi-crystal), then this calculation returns an average value of one that does not decay as \( r \) increases. By contrast, if the superstructural sets do not have the same set of angles, then the value of the MSO-PCF is less than one. Typically, for a system experiencing thermal fluctuations, there will be distributions of the superstructural angles which can have significant widths, and this can lead to decays in the MSO-PCF as a function of \( r \). If desired, further averaging can be achieved by calculating the MSO-PCF for a set of discrete time instants and then averaging the results together to obtain a time-averaged MSO-PCF. While some ensemble-averaging is implied in the above equation, a higher degree of ensemble averaging can be achieved by obtaining independent configurational data for an entirely different region in the sample material at identical conditions. Although not explicitly apparent from the simplified notation, the MSO-PCF for a given material can depend on its composition and density.

As an example, we calculate the MSO-PCF of a particular superstructure of motifs in the Penrose P2 QC pattern at a time \( t = 24 \text{ h}, 48 \text{ h}, 72 \text{ h}, \) and \( 96 \text{ h} \) after release and equilibration. A local motif is first defined by \( m = 5 \) kites, which are arranged into a compact and convex pentagonal star as shown in Fig. 4.6d. In certain instances within the P2 pattern, a central pentagonal-star-kite motifs is surrounded by \( n = 5 \) external pentagonal stars that are not immediately adjacent (see Fig. 4.6d). The coordinates of the center of the motif are obtained directly at single pixel resolution from a digital micrograph taken at time \( t \). We define a first set of five superstructural angles associated with an average first motif set position \( r_a \) (first set: black circles in Fig. 4.6d). Likewise, we define a second set of 5 motif superstructural angles at a
different average second motif set position \( r_b \) using the same procedure (second set: red circles in Fig. 4.6d). We then use Eq. 4.1 to calculate a first contribution to \( G_{5}^{\text{MSO}} \), part of the average denoted by the brackets, and repeat this process over all pairs of superstructural sets in the image, keeping track of each contribution and its associated center-to-center separation. We then bin all of these results according to \( r \) and calculate the average \( G_{5}^{\text{MSO}} \) within each bin interval, displaying this average \( G_{5}^{\text{MSO}} \) at the average \( r \) associated with a particular bin. To examine if there is any temporal evolution, we analyze 4 different micrographs at \( t = 24 \) h, 48 h, 72 h, and 96 h after release using a binning interval of 50 \( \mu \)m and plot average \( G_{5}^{\text{MSO}} \) as a function of \( r \), as shown in Fig. 4.6e.

### 4.2.7 Measuring Area Fractions of Penrose Tiles

The interiors of all tiles in an optical micrograph are filled with colored pixels using an edge-detection routine (i.e. paint bucket fill tool) in Photoshop, and a first estimate of the area fraction is determined by counting these colored pixels and dividing by the total number of pixels. The area fraction of tiles from optical micrographs prior to release is \( \phi_{A,\text{OM}} = 0.677 \pm 0.009 \). This value is \( \approx 13\% \) lower than the designed area fraction of 0.78 on the mask due to optical diffraction during the lithographic printing process and to the dose-response of the SU-8 photoresist. To convert this first estimate into better estimate of the actual measured area fraction, we next use a high resolution SEM image, which does not suffer from optical diffraction, to determine the area fraction of tiles, measured at their upper surfaces, \( \phi_{A,\text{SEMtop}} \). A SEM image (magnification 1000x) of the tops of pre-assembled SU-8 tiles after lithographic exposure and development gives \( \phi_{A,\text{SEMtop}} = 0.684 \pm 0.003 \). The ratio given by \( \phi_{A,\text{SEMtop}} \) divided by \( \phi_{A,\text{OM}} \) is a correction factor \( f_{\text{SEMtop-OM}} = 1.01 \) corrects the uncertainties resulting from optical diffraction and edge of particles in the optical microscopy micrographs.
Since the SEM images of particles before release can only provide areas of the tops of the tiles, these images cannot reveal protruding features on the sides of the particles that are edge roughness, a natural consequence of the photolithographic exposure and development processes. However, such edge roughness can affect the steric interactions between adjacent tiles. To provide a measured area fraction that could be best compared to simulations of tiles that have hard interactions that could potentially be made, we use side or oblique SEM imaging of tiles that have been purposefully lifted off of the substrate following release to estimate the average edge roughness ($\approx 180$ nm). From these SEM measurements, we determine a correction factor of the area fraction as a consequence of this edge roughness to be $f_R = 1.13$. Combining these two correction factors, one from image analysis of optical micrographs and the other from the roughness of particles, the reported measured area fraction, $\phi_M$, can be determined from the filled tiles in the optical micrographs: $\phi_M = f_{\text{SEMtop-OM}}f^* R \phi_{A,OM}$. Thus, the $\phi_M$ reported in the main text includes all corrections for low-dose energy, uncertainties of edge diffraction, and the edge roughness, yielding a best estimate of the area fraction of tiles that potentially can be potentially compared to future simulations of Brownian kite and dart tiles arranged into Penrose P2 patterns.

4.3 Results

To make Litho-PAMs (see Materials and Methods, see section 4.2), we combine computer-aided design software and lithography to produce many shape-designed colloidal particles as well as to position and orient these particles relative to each other in a desired complex initial configuration, even at high particle densities. After lithographic printing, we obtain a pre-assembled set of discrete polymeric particles (i.e. kite and dart "tiles" $\approx 2 \mu \text{m}$ thick in a Penrose P2 QC pattern), composed of crosslinked SU-8 photoresist, that are attached to a $\approx 10$ nm thick layer of water-soluble Omnicoat release material on a glass wafer (Fig. 4.1a). Optical
and scanning electron micrographs confirm that the printed kite and dart tiles form an ideal Penrose P2 QC at a tile area fraction of $\phi_d \approx 0.78$ (Fig. 4.1b). Around this printed region, we build enclosing poly-dimethylsiloxane (PDMS) elastomeric walls, and we add a customized release solution-dispersion (RSD) that we have specially formulated to maintain an intact monolayer of fluctuating tiles that have effectively hard interactions. The RSD contains a base to dissolve the Omnicoat release layer, a surfactant sodium dodecyl sulfate (SDS) to stabilize the released SU-8 particles against aggregation and sticking to the glass substrate, and an appropriately sized depletion agent (e.g. poly-styrene spheres) to create anisotropic roughness-controlled depletion attractions. Because concentration gradients in a rapidly dissolving release material can drive flows that cause tiles to lift off vertically out of the monolayer, we have adjusted the concentration of the base TMAH to be low so the release is gradual. The SDS concentration is raised to provide excellent stability against aggregation, yet is kept low enough that SDS micelle formation is minimal. Moreover, the volume fraction of the depletion agent has been set to be high enough that each tile experiences a facial depletion attraction with the smooth glass substrate that is substantially larger than $k_B T$, yet in-plane depletion attractions with other tiles are much smaller, since edges of tiles are rougher than their faces. Prior attempts to lift-off tiles in a manner that yields a fluctuating Brownian monolayer have failed because they have not incorporated all of these functional additives in the RSD.

After adding the RSD to the PDMS well to begin releasing the tiles (Fig. 4.1c), the glass wafer is positioned on an inverted optical microscope, and the tiles are imaged as they lift off. As the release layer dissolves, previously static tiles become mobile and undergo Brownian motion, frequently colliding with neighboring tiles as the system equilibrates at fixed $\phi_d$ (Fig. 4.1d). Time-lapse digital microphotography yields high resolution movies of the dynamics of release.
By digitally subtracting the initial image before release from images at later times $t$ after the RSD was added, we measure the fraction of tiles that have been released $P_r(t)$ (Fig. 4.1e). We find that this release profile is consistent with first-order reaction kinetics, $P_r(t) = 1 - \exp(-t/\tau)$, where $\tau \approx 1.6$ h is the characteristic time scale of release (solid line, Fig. 4.1e). Thus, >99% of tiles are released after $\approx$8 h, and essentially all particles in the die are released after $\approx$20 h. Although the depletion agent does strongly inhibit tiles from rising above the monolayer, rarely, a very strong Brownian fluctuation well beyond the mean can sometimes cause a tile to vertically rise out of the monolayer, so a few defects can be seen for $t > 72$ h. Thus, $t \approx 48$ h represents a time well beyond release so equilibration has been reached after many interparticle collisions, yet before these few defects appear. Despite Brownian excitations, $\phi_A \approx 0.78$ is large enough that different superstructures of kite-dart motifs remain intact long after release over large length scales (see Fig. 4.2), and the system of fluctuating tiles does not melt.
Figure 4.1 Creating a fluctuating Brownian quasi-crystal of mobile microscale Penrose kite and dart tiles in a confined 2D monolayer. (a) Schematics of Penrose tiles and a pentagonal boundary composed of SU-8 photoresist on a glass wafer. The pentagonal boundary has an inner edge length of 4.5mm. Inset: Scanning electron microscope (SEM) image of a fabricated kite particle (scale bar: 5 µm). All tiles are 2 µm thick. (b), Optical micrograph of pre-configured kite and dart Penrose tiles in an ideal quasi-crystalline pattern at \( \phi_L \approx 0.78 \) after development (scale bar: 10 µm). The right-half of the image is the SEM image with continued QC patterns from the left-half image. (c) Schematics of Penrose tiles in a release solution-dispersion. The RSD is confined in solid poly-dimethylsiloxane (PDMS) walls and covered with a glass cover slip to prevent evaporation. Inset: SEM image of a fabricated dart particle (scale bar: 5 µm). (d) Optical micrograph: 48 hours after adding the solution-dispersion. The right-half of the image has been color-coded via post-acquisition digital analysis: kite (blue) and dart (red). Scale bar: 10 µm. (e) Released fraction of mobile particles, \( P_r \), as a function of time \( t \). Solid red line: fit to \( 1 - \exp(-t/\tau) \), assuming 1st-order reaction kinetics, yielding release time constant \( \tau = 5680 \) s. Inset: schematic side views before and after release. The release layer (green) is dissolved, and the released tiles exhibit Brownian fluctuations in a fully submerged monolayer just above the glass surface, which is also negatively charged, thereby maintaining a lubricating layer of water between tiles and the glass.
Figure 4.2 Examples of organized superstructural sets of motifs extending over different length scales identified in the P2 quasi-crystal before and after release. (a) Regular center-filled pentagonal superstructural set of six 10 kite-5 dart wheel motifs (kites filled blue, darts filled red) before release (0 h, leftmost), and after release (6, 12, 24, 26, 48 h from left to right). (b) Regular decagonal superstructural set of ten 10 kite-5 dart wheel motifs before release (0 h, leftmost) and after release (12, 24, 48 h from left to right). (c) Regular icosagonal (i.e. 20-sided polygon) superstructural set of twenty 10 kite-5 dart wheel motifs before release (0 h, left) and after release (48 h, right). Scale bar shown in (a) is the same for all images: 20 µm.

To determine how Brownian fluctuations of mobile P2 tiles can alter the degree of quasi-crystalline order compared to static tiles, we take Fourier transforms (FTs) of optical micrographs to produce the equivalent of light scattering intensity patterns. Interiors of tiles are digitally filled black to enhance contrast. The nearly perfect QC order before release (Fig. 4.3a) yields Bragg-like peaks at low scattering wavenumbers $q$, indicative of ordering of larger
superstructures of tiles, and also 10 narrow rays extending from the center towards high $q$, indicative of the high degree of 5-fold orientational order of the edges of all tiles (Fig. 4.3b).

Expanded views at lower $q$ and very low $q$ (Fig. 4.3c: $\approx 6x$ and Fig. 4.3d: $\approx 12x$) reveal sets of 10 Bragg peaks, demonstrating pentagonal QC ordering of motifs and superstructures of motifs over long-range. After release and equilibration, mobile Brownian kite and dart tiles at a given instant no longer have positions and orientations corresponding to an ideal Penrose P2 tiling (Fig. 4.3e). Entropic Brownian fluctuations destroy ideal QC order; tiles no longer have perfect positions and orientations on a QC lattice, yet the overall QC morphology is preserved. Thus, the Brownian fluctuations have caused the 10 rays at high $q$ to broaden azimuthally (Fig. 4.3f). At intermediate $q$, Bragg peaks have disappeared and instead 10-fold modulations in ring-like intensities are seen (Fig. 4.3g, $\approx 6x$), reminiscent of 6-fold modulations seen in hexatic liquid-crystal-like systems. Interestingly, large superstructures of motifs still retain considerable spatial and orientational order, since Bragg peaks are still observed at the very lowest $q$ (Fig. 4.3h, $\approx 12x$). All but the superstructural peaks at the very lowest $q$ have become smeared out, leaving only 10-fold azimuthal intensity modulations at intermediate and high $q$.

Entropic-thermal line broadening is very evident at high $q$, so we fit the $\psi$-dependent azimuthal intensity, $I(\psi)$, of an emanating ray using a single Gaussian function with a constant background (Fig. 4.3i): $I(\psi) = I_0 + I_h \exp[-(\psi-\psi_p)^2/(2\psi_\sigma^2)]$, where $I_0$ is the background intensity, $I_h$ is the peak intensity relative to $I_0$, $\psi_p$ is the peak angle, and $\psi_\sigma$ is the standard deviation in angle (i.e. effective azimuthal width of a ray). A fit to the data in Fig. 4.3i before release yields $I_0 (10^9) = 1.48 \pm 0.13$, $I_h (10^9) = 11.5 \pm 0.3$, $\psi_p = 50.6 \pm 0.1$ deg, and $\psi_\sigma = 2.6 \pm 0.1$ deg with a
correlation coefficient $R^2 = 0.996$. A fit to the data in Fig. 4.3i after release yields $I_0 (10^9) = 0.45 \pm 0.09$, $I_h (10^9) = 1.70 \pm 0.11$, $\psi_p = 50.5 \pm 0.3$ deg, and $\psi_\sigma = 5.4 \pm 0.5$ deg with a correlation coefficient $R^2 = 0.977$. Thus, entropic fluctuations after release cause a noticeable increase in the width $\psi_\sigma$ by $\approx 2x$ and decrease in the central intensity of the rays at high $q$ by $\approx 7x$.

At intermediate $q$, we fit $I(\psi)$ before release with a double Gaussian function to capture the Bragg-like peak and after release with a single Gaussian function (Fig. 4.3j). In Fig. 4.3j, corresponding to the FT before release, we semi-empirically fit the azimuthal line shape at lower $q$ using a double Gaussian function: $I_0 + I_{h,1} \exp[-(\psi-\psi_p)^2/(2\psi_\sigma^2)] + I_{h,2} \exp[-(\psi-\psi_p)^2/(2\psi_\sigma^2)]$, obtaining $I_0 (10^{11}) = 1.76 \pm 0.18$, $I_{h,1} (10^{11}) = 8.2 \pm 1.6$, $\psi_p = 50.7 \pm 0.1$ deg, $\psi_\sigma = 1.03 \pm 0.21$ deg, $I_{h,2} (10^{11}) = 5.5 \pm 1.6$, and $\psi_\sigma = 3.18 \pm 0.53$ deg with a correlation coefficient $R^2 = 0.996$. The double Gaussian function is necessary to fit the high Bragg-like peak; a single Gaussian function cannot adequately capture this sharp peak. In Fig. 4.3j, after release, the sharp peak has disappeared, and so we fit the azimuthal line shape at low $q$ using only a single Gaussian function: $I_0 + I_h \exp[-(\psi-\psi_p)^2/(2\psi_\sigma^2)]$, yielding $I_0 (10^{11}) = 0.46 \pm 0.19$, $I_h (10^{11}) = 1.72 \pm 0.18$, $\psi_p = 51.2 \pm 0.4$ deg, and $\psi_\sigma = 6.8 \pm 1.0$ deg with a correlation coefficient of $R^2 = 0.96$. Thus, the very sharp, Bragg-like nature of this peak has dramatically changed as a consequence of the entropic fluctuations of the kite and dart tiles in the Brownian P2 system. This disappearance of Bragg peaks, yet preservation of a 10-fold modulation in $\psi$ at this $q$ indicates that motifs of tiles are no longer spatially ordered, but the majority of motifs do preserve a significant degree of
orientational order. Similar peak and line broadening effects after release are also evident in FTs of only kite tiles (Fig. 4.4) and only dart tiles (Fig. 4.5).

Figure 4.3 Entropic restructuring of ordered Penrose kite and dart tiles into a fluctuating liquid quasi-crystal (LQC) monolayer after release. (a) Optical micrograph of Penrose QC tiles before release (interiors of tiles are filled black to enhance contrast). Scale bar: 20 µm. (b) Effective scattering pattern given by the Fourier transform (FT) intensity of (a) showing 10 rays extending from the center to high scattering wavenumbers q. (c) Central region of (b), zoomed by ≈6x, revealing Bragg peaks at intermediate and low q. (d) Central region of (c), zoomed by ≈2x, revealing Bragg peaks at very low q. (e) Optical micrograph of fluctuating Penrose QC tiles 48 hours after release (tile interiors filled black). (f) Average of FFT intensities of three different micrographs taken at 46, 48 (see (e)), and 50 hours after release. (g) (h) Zooms of (f) over the same q ranges as in (c) and (d), respectively. (i) High-q azimuthal line shapes I(ψ) of emanating rays (see inset defining ψ and q: white dashed lines), before release (black circles) and after release (blue triangles). Lines are fits to Gaussian functions (see text). (j) Intermediate-q I(ψ) (see inset defining ψ and q: white dashed lines), before release (black circles) and after release (blue triangles). Black line: fit to a double Gaussian; blue line: fit to a Gaussian (see text). (k) Intensity scale bar for all FTs shown.
Figure 4.4 Penrose kite tiles in a P2 quasi-crystal before release (a-d) and after release (e-h). (a) Kite tiles are separated by micrograph image post-processing, from Fig. 4.3a. Scale bar (lower right): 20 µm. (b) Effective scattering pattern, given by the Fourier transform (FT) intensity of (a), showing 10 rays extending from the center to high scattering wavenumbers q. (c) Central region of (b), zoomed by ≈6x, revealing Bragg peaks at intermediate and low q, corresponding to large distances (d) Central region of (c), zoomed by an additional ≈2x, revealing Bragg peaks at very low q associated with superstructural ordering of motifs of kite tiles over large length scales. (e) Brownian fluctuations near equilibrium (48 h after release) have caused kite tiles to deviate from the original perfect QC order seen in the unreleased structure; kites are separated from Fig. 4.3e. (f) FT intensity of (e). Rays have broadened azimuthally as a consequence of Brownian fluctuations. (g), (h) Zooms of (f) over the same q ranges as in (c) and (d), respectively, showing the smearing of Bragg peaks into 10-fold azimuthal intensity modulations, reminiscent of liquid-crystalline materials, indicating a retention of quasi-crystalline orientational order. Color scale bar of intensity is the same as in Fig. 4.3k.
Figure 4.5 Penrose dart tiles in a P2 quasi-crystal before release (a-d) and after release (e-h). (a) Dart tiles are separated by micrograph image post-processing, from Fig. 4.3a. Scale bar (lower right): 20 µm. (b) Effective scattering pattern, given by the Fourier transform (FT) intensity of (a), showing 10 rays extending from the center to high scattering wavenumbers $q$. (c) Central region of (b), zoomed by $\approx 6x$, revealing Bragg peaks at intermediate and low $q$, corresponding to large distances. (d) Central region of (c), zoomed by an additional $\approx 2x$, revealing Bragg peaks at very low $q$ associated with superstructural ordering of motifs of dart tiles over large length scales. (e) Brownian fluctuations near equilibrium (48 h after release) have caused dart tiles to deviate from the original perfect QC order seen in the unreleased structure; darts are separated from Fig. 4.3e. (f) FT intensity of (e). Rays have broadened azimuthally as a consequence of Brownian fluctuations. (g), (h) Zooms of (f) over the same $q$ ranges as in (c) and (d), respectively, showing a smearing of Bragg peaks into 10-fold azimuthal intensity modulations, reminiscent of liquid-crystalline materials, indicating a retention of quasi-crystalline orientational order. Color scale bar of intensity is the same as in Fig. 4.3k.

Equilibrium Brownian forces cause multi-tile motifs, such as the 15 kite-5 dart pentagonal flower (Fig. 4.6a, top) and the 10 kite-5 dart pentagonal wheel (Fig. 4.6a, bottom), to distort randomly and to break chiral symmetry locally without melting. Within the fluctuating P2 system, we observe that 5 kites in pentagonal-star-kite motifs (PSKMs) can rotate collectively, and the entire motif makes rotational transitions between different preferred angles, defined by corrugations in the fluctuating structures outside (Fig. 4.6b, top). We measure the heterogeneous hopping dynamics of these collective rotational fluctuations by digitally tracking the rotational...
angle $\alpha$ and trajectory of the centroid of one kite in the PSKM over time (Fig. 4.6b, bottom; Fig. 4.6c). Although individual motifs can distort and some can even rotate, larger scale superstructures made up of multiple motifs appear to preserve at least orientational order over long range. To quantify this, we have developed a motif superstructural orientational pair correlation function (MSO-PCF), $g_{MSO-PCF}$, that can depend on the separation $r$ between the centers of two similar superstructures of motifs. For instance, we define a superstructure of five PSKMs in a nearly regular pentagonal configuration that surround a central PSKM (Fig. 4.6d); superstructural bond angles (dashed lines, Fig. 4.6d) are then correlated in a manner analogous to bond-orientational pair correlation functions of individual particles. Before release, $g_{MSO-PCF}(r)$ is 1, whereas after release $g_{MSO-PCF}(r)$ exponentially decays from 1 down to a plateau that is just below unity, indicating strong preservation of relative superstructural bond angles over long range (Fig. 4.6e). The high and persistent value of $g_{MSO-PCF}$ at large $r$ over long times indicates long range orientational order of superstructures of motifs persists in equilibrium after release even as tile-tile spatial order is destroyed at long range as a consequence of entropic Brownian fluctuations of all tiles. Thus, MSO is preserved over long times even as distortions and rotations of smaller motifs occur. Moreover, because the system of mobile tiles exhibits solid-like behavior, similar to simpler dispersed systems of colloidal hard spheres at high densities, we observe entropically generated sound wavelets that scatter locally and are damped.
Figure 4.6 Motif dynamics and superstructural orientational pair correlation function. (a) Optical micrographs of fluctuating pentagonal motifs of Penrose kite-and-dart tiles before release and 12, 24, 36, and 48 hours after release: 15 kite-5 dart flower (upper row); 10 kite-5 dart wheel (lower row). Scale bar: 10 µm. (b) Collective rotational fluctuations of a central pentagonal star of kites (black outlines) within a flower over time. (scale bar: 10 µm) (c) Trajectory of the centroid of the color-coded kite shown in (b), which displays heterogeneous dynamics. (scale bar: 3 µm) (d) A first pentagonal superstructure of pentagonal star-kite motifs (PSKMs, kites filled blue, black dot at center) is separated by a center-to-center distance $r$ from a second pentagonal superstructure of PSKMs (kites filled blue, red dot at center). Solid green arrow: vector between centers of superstructures. Dashed green arrows: vectors between centers of superstructures and constituent PSKMs. (scale bar: 20 µm) (e) Motif superstructural orientational pair correlation function (MSO-PCF), $g_{MSO-PCF}$, as a function of a $r$: 24 (black circles), 48 (blue squares), 72 (red diamonds), and 96 hours (green triangles) after release. Solid red line: fit to the average of 48 and 72 hour data using exponential decay of $g_{MSO-PCF}$ from 1 down to a plateau of $g_{MSO-PCF} = 0.96$ at large $r$. 

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Beyond studying equilibrium fluctuations a high $\phi_A$, we also cause dense P2 patterns to melt by removing the confining walls to slowly reduce $\phi_A$. In a recorded time-lapse movie, entropic-Brownian forces cause tiles to diffuse into the available empty space over time, thereby creating a gradient in $\phi_A$ (Figs. 4.7a-4.7c, empty space towards right of images). Digitally color-coded pentagonal kite motifs (blue) and pentagonal dart motifs (red) progressively melt over time; the melting front moves towards the left. Over time, we determine $\phi_A$ of all tiles (Fig. 4.7d) as well as the area fractions of pentagonal kite motifs, $\phi_{A,KM}$ (Fig. 4.7e) and of pentagonal dart motifs as a function of distance $d$ measured from the left. Progressive melting of both types of motifs is observed, as $\phi_A$ is lowered and the melting front moves from right to left. We fit these measured $\phi_A(d)$ using a modified Fermi-like function (Fig. 4.7d and 4.7e, solid lines) to capture the evolution of diffusive dynamics associated with melting as the Brownian P2 QC expands to occupy empty space after removing a confining wall. This function can be expressed as: $\phi_A(d) = \phi_A^*/(1+\exp[(d-d_0)/L])$, where $\phi_A^*$ is a plateau area fraction in the dense region (to the left), $d_0$ is a reference distance that roughly defines the position of the melting front, and $L$ is a characteristic length scale associated with the width of the melting front. We use similar Fermi-like fitting forms to fit data for the $d$-dependent area fractions of pentagonal-star-kite motifs, $\phi_{A,KM}$, and pentagonal-star-dart motifs, $\phi_{A,DM}$. All fitting parameters for $\phi_A$ (Fig. 4.7d), $\phi_{A,KM}$ (Fig. 4.7e), and $\phi_{A,DM}$ (Fig. 4.8), as a function of time after release, $t$, are shown in the table 4.1. FTs of the same region roughly before, during, and after the passage of the melting front (Figs. 4.7f-4.7h, respectively) reveal the progressing disappearance of spatial and orientational order. Bragg-like peaks at very low $q$ which are evident before melting (Fig. 4.7f) disappear, and only glassy-like isotropic rings remain in the effective scattering pattern immediately after melting (Fig. 4.7h). To
determine $\phi_A$ associated with motif melting, we eliminate $d$ and plot $\phi_{A,KM}$ versus $\phi_A$ at different times $t$ (Fig. 4.7i). In Fig. 4.7i, we fit all data for $\phi_{A,KM}(\phi_A)$ satisfying $\phi_{A,KM} > 0$ to a linear function having a slope and an intercept with the $\phi_A$ (i.e. horizontal) axis. The slopes of $\phi_{A,KM}(\phi_A)$ for 24, 48, and 60 h after release are $1.30 \pm 0.04$, $2.26 \pm 0.14$, and $2.42 \pm 0.53$, respectively, and the intercepts with the $\phi_A$-axis are $0.544 \pm 0.006$, $0.633 \pm 0.004$, and $0.647 \pm 0.009$, respectively. At $t = 48$ h and $t = 60$ h after release, these intercepts are approximately the same within the uncertainties, so we estimate that the melting point of the P2 quasi-crystal is at $\phi_{A,melt} \approx 0.65 \pm 0.02$. While this value is associated with the melting of pentagonal-star-kite motifs, larger scale superstructures of motifs become ill-defined when the fundamental motif structures become disorganized. Dart motifs also melt at a similar $\phi_{A,m}$ (Fig. 4.8).
Figure 4.7 Entropic melting dynamics of an unconfined fluctuating Brownian quasi-crystal. Color-coded optical micrograph of Penrose kite-and-dart tiles that diffusively melt into an unconfined open region beyond the right of the micrograph at different times after release: (a) 4, (b) 24, and (c) 48 h. release (blue: pentagonal star-kite motifs, red: pentagonal star-dart motifs). (d) Area fraction of Penrose kite-and-dart tiles, $\phi_A$, as a function of a distance, $d$, starting from the left of the top micrograph images at different times after release: 4 h (black circles), 24 h (orange diamonds), and 48 h (purple triangles) hours release. Lines are fits to an empirical Fermi function (see Methods). (e) Area fraction of unmelted PSKMs, $\phi_{A,KM}$, as a function of $d$ at different times after release: 4 h (black circles), 24 h (orange diamonds), and 48 h (purple triangles). Lines are fits to an empirical Fermi function (see Methods). (f) Fourier Transform (FT) of image (a)-inside yellow dashed lines. 10-fold azimuthal intensity modulations are evident. (g) FT of image (b)-inside yellow dashed lines. 10-fold azimuthal intensity modulations are less
pronounced. (h) FT of image (c)-inside yellow dashed lines. 10-fold azimuthal intensity modulations are not visible, and the ring-like pattern indicates that tiles in the region are largely disordered as in a glass. (i) To determine the melting area fraction $\phi_{A,melt}$ of the P2 QC, we plot $\phi_{A,KM}$ as a function of $\phi_A$ at times after release: 24 h (green circles), 48 h (orange diamonds), and 60 h (purple triangles). At the two longest times, we find nearly the same motif melting behavior, yielding an intercept with the horizontal axis of $\phi_{A,melt} \approx 0.65$. Lines: linear fits (see Methods).

![Graph showing the melting of pentagonal-star-dart motifs](image)

**Figure 4.8 Melting of pentagonal-star-dart motifs in an unconfined Brownian P2 quasi-crystal.** The area fraction of pentagonal-star-dart motifs, $\phi_{A,DM}$, (red darts in Fig. 4.7) decays to zero at large distances $d$, towards the direction where the quasi-crystal is not confined. The motif melting front moves from left to right (i.e. toward the direction of open space) over time: black circles (4 h after release), orange diamonds (24 h), and purple triangles (48 h). Fits are to a Fermi-like function, and the fit parameters are displayed in Table 4.1.

**Table 4.1** Fitting parameters of Fermi-like function for $d$-dependence of $\phi_A$, $\phi_{A,KM}$, and $\phi_{A,DM}$. $R^2$ is the correlation coefficient of the fit. The time after initiation of release is $t$.

<table>
<thead>
<tr>
<th>Data Fit</th>
<th>Fitting Parameter</th>
<th>$t = 4$ h</th>
<th>$t = 24$ h</th>
<th>$t = 48$ h</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_A(d)$</td>
<td>$\phi_A^*$</td>
<td>0.770 ± 0.001</td>
<td>0.768 ± 0.003</td>
<td>0.762 ± 0.002</td>
</tr>
<tr>
<td>$d_0 (\mu m)$</td>
<td>846 ± 16</td>
<td>744 ± 9</td>
<td>773 ± 5</td>
<td></td>
</tr>
<tr>
<td>$L (\mu m)$</td>
<td>98 ± 7</td>
<td>95 ± 7</td>
<td>162 ± 5</td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.994</td>
<td>0.994</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>$\phi_{A,KM}(d)$</td>
<td>$\phi_{A,KM}^*$</td>
<td>0.314 ± 0.003</td>
<td>0.285 ± 0.005</td>
<td>0.269 ± 0.013</td>
</tr>
<tr>
<td>$d_{0,KM} (\mu m)$</td>
<td>750 ± 35</td>
<td>567 ± 6</td>
<td>387 ± 16</td>
<td></td>
</tr>
<tr>
<td>$L_{KM} (\mu m)$</td>
<td>79 ± 22</td>
<td>49 ± 6</td>
<td>66 ± 13</td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.921</td>
<td>0.989</td>
<td>0.981</td>
<td></td>
</tr>
<tr>
<td>$\phi_{A,DM}(d)$</td>
<td>$\phi_{A,DM}^*$</td>
<td>0.058 ± 0.001</td>
<td>0.062 ± 0.001</td>
<td>0.029 ± 0.003</td>
</tr>
<tr>
<td>$d_{0,DM} (\mu m)$</td>
<td>691 ± 7</td>
<td>517 ± 5</td>
<td>348 ± 29</td>
<td></td>
</tr>
</tbody>
</table>
4.4 Discussion

Although a range of top-down lithographic methods have been used to produce custom shape-designed colloidal particles\textsuperscript{32-34}, these processes typically yield stable bulk dispersions of desired shapes in a liquid after lifting them off of a substrate. Subsequently, these shapes have been used in depletion-driven and capillary-driven self-assembly experiments, providing new insights into how features in the shapes of particles can influence self-assembly. For instance, lithographic mutations of sub-particle features of colloidal chiral C-shapes that resemble proteins, known as proteoids, have been used to control the entropic hierarchical self-assembly of dimer crystals under slow crowding in 2D as roughness controlled depletion attractions keep the fluctuating monolayer intact\textsuperscript{35}. Nevertheless, the sizes of crystallites of such self-assembled structures are typically small, and a high defect density often accompanies self-assembly. Although these studies have provided important scientific insights into the role of core shape and entropy in self-assembly of gradually crowded systems, including protein crystallization, these limitations have precluded the widespread use of such self-assembled structures. Thus, alternative experimental methods for creating defect-free, highly complex, multi-scale structures of mobile shapes over large spatial regions are needed. Our work demonstrates that it is possible to produce and study complex fluctuating multi-scale Litho-PAMs of mobile Brownian tiles at high tile densities using optical stepper lithography and optical microscopy.

This pre-assembly method of producing Litho-PAMs is very different than prior 2D self-assembly experiments in which monolayers of shape-designed lithographic colloids were randomly deposited at dilute area fractions in a monolayer and subsequently concentrated slowly using gravitational sedimentation in the presence of roughness-controlled depletion attractions\textsuperscript{36}. This prior approach of concentrating particles actually creates a gradient in particle area fraction
throughout a tilted optical cell; whereas, by contrast, the pre-assembly method that we present here provides a uniform area fraction over a very large surface area. Thus, pre-assembly avoids potential issues of out-of-equilibrium jamming during crowding as well as spatial gradients in $\phi_A$ that were inherent in this past method of 2D self-assembly.

One of the most compelling examples of a complex, multi-scale, hierarchically organized structure is the Penrose P2 kite-dart tiling, an example of a two-dimensional quasi-crystal (QC). Making a monolayer of mobile Brownian kite and dart tiles in Penrose's P2 pattern that fluctuate entropically, that are defect-free over large areas, and that can be readily visualized, advances the experimental science of mobile multi-scale structures, and demonstrates that new equilibrium phases composed of many differently shaped and configured building blocks can be readily produced using top-down parallel fabrication methods, thereby circumventing bottom-up self-assembly methods and serial directed assembly methods. For systems that can have degenerate ground states with the same free energy yet different polymorphic organizations of tiles, Litho-PAMs even enables the creation and study of different individual ground-state configurations. Such degeneracy would normally preclude simple self-assembly methods from producing different desired ground-state configurations, since different ground-state polymorphs having essentially the same energy would nucleate and grow in different local regions.

Until now, attempts to create a large-scale fluctuating Penrose QC of mobile Brownian colloidal tiles, which are organized with 5-fold symmetry and which have effectively hard interactions, have not succeeded. While a report of a soft-matter QC of nanoparticles which were crowded through evaporation has been made previously, the crystallites in this self-assembled QC were limited to 12-fold\textsuperscript{37,38} and 18-fold symmetry\textsuperscript{38}. Even after this report, the unusual and striking 5-fold symmetry of Penrose's P2 QC\textsuperscript{39} has remained elusive, and no experimental
assembly method has yet been able to produce a fluctuating colloidal version of it until now. Even if in principle a Brownian Penrose QC can be self-assembled by introducing selective site-specific edge-edge interactions among the tiles, as has been proposed and investigated in simulations\textsuperscript{40,41}, no experimental realization of this has yet been made, most likely because of the significant complexity in creating the required variety of selective edge-edge interactions on real particles. By contrast, the pre-assembly approach of Litho-PAMs overcomes these significant limitations, and has enabled us to produce a fluctuating P2 QC of differently shaped Brownian tiles having 5-fold symmetry at high densities in a single 2D large-area domain. Moreover, we have visualized the equilibrium fluctuations of this Brownian P2 QC at high tile densities, including heterogeneous collective dynamics of certain motifs, as well as melting by slowly lowering the tile density. Our ability to observe mobile tiles fluctuating in equilibrium using optical microscopy has enabled us to identify the hallmarks of the pentatic liquid quasi-crystalline (LQC\textsubscript{5}) phase of matter, and this provides important in situ dynamic information that cannot be obtained by studying a static solid film using electron beam microscopy after evaporating the continuous liquid phase.

Interesting fundamental questions about spatio-temporal dynamics arise when considering complex multi-scale materials composed of mobile building blocks, such as the P2 kite-dart system. Traditional models of phase behavior and equilibrium fluctuations, such as cage models based on local groups of particles, do not necessarily transfer over to multi-scale systems that have a great variety of dynamic fluctuating motifs and patterns that can be hundreds or more times in overall size compared to the smallest particles. Understanding equilibrium and non-equilibrium dynamics of multi-scale systems that have differently organized structures at different length scales, including those well beyond the colloidal scale, remains an interesting
and challenging theoretical frontier. For example, directly applying the notions of Kosterlitz-Thouless (KT) theory\textsuperscript{42} to a fluctuating P2 quasi-crystal is a challenging problem, yet based on our observations the notions of KT theory when applied to fluctuating quasi-crystals are likely to show the emergence of liquid crystalline like features in correlation functions and order parameters at smaller length scales for values of $\phi_A$ in between the disordered liquid state at low $\phi_A$ and the full-tiling limit as $\phi_A$ approaching to 1. Just as disks can form a hexatic liquid crystal phase over a certain range of $\phi_A$\textsuperscript{43}, our observations indicate that liquid-crystal like modulations in the FTs develop for fluctuating Brownian P2 quasi-crystals at intermediate wavenumbers. Yet, we also find that the degree of fluctuation-induced smearing of QC Bragg peaks at the smallest wavenumbers $q$, corresponding to the very largest length scales, is not as large as the degree of fluctuation-induced smearing at intermediate $q$. The $g_{\text{MSO-PCF}}(r)$ that we have developed is a first attempt at quantifying superstructural order in hierarchically organized multi-scale materials, and this concept can be further broadened and generalized. This brings up an important theoretical question for multi-scale materials that can have different symmetries and potentially incommensurate organizational structures at different length scales: what is truly meant by short, intermediate, and long range order? Based even on the example of a fluctuating P2 QC, we believe that it may be necessary to correlate the type and degree of order with a range of length scales (or equivalently range of $q$) in multi-scale materials, possibly also even spatial locations, and a new theory would seek to couple these differently structured regions together self-consistently. Furthermore, a theoretical exploration of melting in hierarchically organized systems of differently shaped fluctuating building blocks, including kites and darts in the fluctuating P2 QC system, would potentially enable a direct comparison with our experimental results of melting.
We anticipate that Litho-PAMs can be extended to create significantly smaller tiles than those we have first demonstrated using optical UV stepper lithography if the depletion agent in the RSD is correspondingly altered and re-optimized. Spin-coating can produce SU-8 layers having uniform thickness down to about 10 nm after baking, and advanced steppers can produce ≈10 nm feature sizes over large die areas. So, in principle, tiles that have complex shapes and maximum lateral dimensions down to ≈50 nm and thicknesses down to ≈10 nm could be fabricated using advanced optical lithographic steppers that are currently available. For a 5 cm x 5 cm die region, easily achievable with such advanced steppers, this would correspond to ≈10^{12} tiles (having up to as many different shapes of tiles) in a desired pre-assembled configuration. Adjusting the depletion agent's size and its concentration in the RSD would most likely be necessary in order to maintain an intact mobile monolayer of smaller tiles after release. Likewise, nanoimprint lithography\textsuperscript{44} is a parallel fabrication process that could be used to produce tiles using Litho-PAMs, yet avoiding unwanted bridging artifacts between tiles at high densities over the entire printed area would likely be difficult to overcome. Other forms of nanoscale lithography\textsuperscript{45}, including e-beam, x-ray, dip-pen lithography, could be used to fabricate the tiles at even smaller scales than we have demonstrated, yet these serial writing methods have the drawback of being significantly slower than parallel methods. Overall, the production of dense configurations of lithographically pre-assembled colloidal shapes, which we have illustrated in 2D with Litho-PAMs, can be readily extended to other forms of lithography, including 3D micro- and nano-lithographic methods.
4.5 Conclusions

We have demonstrated that Litho-PAMs can successfully generate extremely complex, human designed, defect-free monolayers of mobile colloidal objects that have effectively hard interactions and fluctuate entropically, as we have shown with kite and dart tiles in a Penrose P2 QC pattern. This lithographic pre-assembly approach, combined with the specially formulated and optimized RSD that maintains the monolayer during and after release, are quite general and open up an enormous range of possible multi-scale monolayer structures that can now be examined. By controlling $\phi_t$ and the degree of confinement of the system, the phase behavior and melting of many interesting 2D systems can now be measured and compared with theories and simulations. We anticipate that this will lead to a broader understanding of heterogeneous dynamics in multi-component, multi-scale Brownian systems. Moreover, interactions between the particles in the monolayer can potentially be tuned by further altering the RSD. While our demonstration of Litho-PAMs has been in 2D, we believe that it will be possible to extend this approach into 3D using lithography techniques such as stereo-microlithography or holographic lithography. By circumventing well-known problems associated with self-assembly, Litho-PAMs can be used to generate a wide range of interesting Brownian systems that can begin to approach the extremely high degree of complexity in biological monolayers and membranes.
References


Chapter 5 – Summary

This dissertation has described the experimental formation and behavior of new organized multi-scale materials that are colloidal monolayers of mobile shape-designed lithographic tile particles. In particular, we have demonstrated that both bottom-up self-assembly and top-down pre-assembly can be useful for creating hierarchically organized 2D systems of dense tiles that fluctuate and interact as a consequence of Brownian motion. We have studied the self-assembly of dimers and dimer crystals made of C-shaped tiles, both achiral and chiral ASPs, in 2D through slow osmotic crowding. Moreover, we have created and studied the first fluctuating 2D Penrose quasi-crystal of kite and dart tiles by developing and successfully demonstrating a new pre-assembly and chemical release process.

As a first major advance, we have lithographically designed and fabricated achiral C-shaped ASPs that have both concave and convex regions; for a certain range of opening angles, ASPs have the capacity to form mutual lock-and-key dimers. To study dimerization of such ASPs at high densities, we have created Brownian monolayers of them in a manner that ensures nearly hard in-plane interactions. By creating a monolayer at dilute area fractions and then slowly osmotically compressing this monolayer to cause slow crowding, we show that the achiral ASPs self-assemble into lock-and-key dimers having equal 50:50 proportions of positive and negative chiralities at high densities. Because achiral C-shapes form a racemic mixture when slowly crowded, dimer crystallization is suppressed and a glassy-like disordered jammed state forms. Moreover, we have measured the osmotic equation of state given by the 2D osmotic
pressure as a function of area fraction, $\Pi_{2D}(\phi_d)$, of a 2D achiral ASP system, yielding the exponential connection between the equilibrium constant $K$ associated with dimerization and $\Pi_{2D}$.

Motivated by dimer crystallization of chiral proteins, we have modified the shapes of achiral ASPs to suppress sterically one chiral dimerization pathway and select only the other pathway. An enantiopure dimer system could have a much greater proclivity to form dimer crystals. So, we have created systems of lithographically fabricated proteomimetic colloids, or 'proteoids', and have explored how Brownian monolayers of mobile proteoids, which have hard interactions, self-assemble when these proteoids are slowly crowded. Remarkably, chiral C-shaped proteoids having circular heads on only one side form enantiopure lock-and-key chiral dimers; these dimers have corrugated, shape-complementary perimeters, so they, in turn, form lock-and-key arrangements into chiral dimer crystals. Time-lapse video microscopy reveals the expulsion of monomers from the growing dimer crystals through tautomerization translocation reactions which expedite the crystallization kinetics. By lithographically mutating proteoids, we also tune the types and primitive vectors of the resulting dimer crystals. Thus, we have shown that rationally designed "lithographic mutations" of sub-particle features in hard-core colloidal shapes can be used to sterically select desired self-assembly pathways without introducing any site-specific attractions, thereby generating a striking degree of hierarchical self-ordering in the absence of site-specific attractive interactions. We believe that such steric pathways and tautomerization translocation reactions could play roles in the dimer crystallization of certain proteins.

Beyond bottom-up self-assembly approaches, we show that large-scale hierarchically organized systems of microscale tiles, such as quasi-crystalline Penrose tilings, can be created in
2D through a highly parallel lithographic printing and release process into a solution-dispersion containing a nanoscale depletion agent. This approach, which we call Litho-PAMs, enables us to control the sizes, shapes, and relative positions and orientations of colloidal building blocks, so that we can produce ground state configurations that are typically inaccessible through self-assembly. As a demonstration of this approach, we have produced a randomly fluctuating Penrose P2 quasi-crystal of mobile kite and dart tiles, and using high resolution time-lapse optical microscopy, we study local and non-local dynamics of particles, motifs, and superstructures. Moreover, by removing a confining wall, we reveal the melting of this fluctuating quasi-crystal through a pentatic liquid quasi-crystalline (LQC) regime. Thus, we have demonstrated a facile and versatile route for creating pre-configured long-lived Brownian monolayers of differently shaped colloidal tiles that have effectively hard in-plane interactions. We anticipate that Litho-PAMs will be used to make and study many other kinds of multi-scale materials.