

Programmable Three-Dimensional Self-Assembly

Will Glazener

Graduate Student Mentor: Ben Cassidy
Faculty Supervisor: Dr. C. Grant Willson

Department of Chemical Engineering
Cockrell School of Engineering
The University of Texas at Austin

Abstract

The development of modern technology has emphasized the utility of nanomaterials and efficient design of nanostructures. Self-assembly is a powerful, bottom-up approach for the fabrication of nanomaterials. The Hammer and Tongs method that we have developed signifies the advent of a holistic approach to programmable, three-dimensional self-assembly. This method integrates biochemistry and chemical engineering techniques to create DNA-mediated, self-assembling polymer cubes. Selective functionalization of cubic faces with complimentary DNA sequences provides a programmable route for self-assembly. The development of this method and its process steps demonstrates the potential to form complex structures on extremely small scales.

Contents

Introduction	4
Theory	8
Creation of Building Blocks	8
Functionalization of Cubes Faces	10
<i>Carbodiimide Coupling</i>	11
<i>Thiol-ene Click Chemistry</i>	14
<i>Azide-alkyne Click Chemistry</i>	14
Hammer and Tongs Method	15
Process Flow	18
Results	20
Conclusion	23
References	24
List of Figures	
Figure 1: SEM Janus Particles	6
Figure 2: Method Process Flow	7
Figure 3: Photolithography	9
Figure 4: Carbodiimide Flow Cytometry	12
Figure 5: Carbodiimide Box Behnken	13
Figure 6: Hammer and Tongs Polymer Stack	16
Figure 7: Hammer and Tongs Post-Etch	17
Figure 8: Photomask Domains	19
Figure 9: Hammer and Tongs Selective Functionalization	20
Figure 10: Overlapping Selective Functionalization	21
Figure 11: Cube Dimers	22

Introduction

Self-assembly is the process by which disordered systems of individual components form more ordered structures without external driving forces.¹ These systems are composed of three key elements: building blocks, associative forces, and dissociative forces. Self-assembling processes are spontaneous, reversible processes that occur when associative forces overcome dissociative forces, and join building blocks together in a higher state of order. The internal driving forces for these processes are the associative forces between building blocks, and can be influenced by changes to system properties, such as thermal energy, presence of chemical species, pressure, and electromagnetic fields.

Self-assembly occurs across all scales of size, including the creation of galaxies and formation of cellular bilayers; however, its applications in nanotechnology have been of particular interest because it provides a bottom-up approach for creating nanostructures.² The two main approaches of nanofabrication are the top-down and bottom-up approaches. In the top-down approach, nanostructures are created from larger structures by removing parts of the structure. This approach is analogous to creating a marble statue by chiseling material away from a larger block. Another example is the current method of semiconductor fabrication, which employs nanolithography as a top-down approach for patterning circuits on silicon chips via photoreactive polymers. In the bottom-up approach, structures are assembled from smaller components, such as cars manufactured on an assembly line. It is this approach that the idea of molecular self-assembly is based on. Molecular self-assembly is the process by which individual molecules arrange themselves in a higher order conformation due to internal associative driving forces.³ By using

individual molecules as building blocks, molecular self-assembly provides a viable method for creating nanostructures. For self-assembly on the nanometer scale, the unique molecular recognition properties of complementary DNA strands are employed as associative driving forces.⁴

Although DNA is commonly thought of as a carrier of genetic information in biological systems, it is employed as a structural material in molecular self-assembly, completely outside of the context of living cells.⁵ Its familiar double-helix structure consists of two complementary strings of single-stranded DNA (ssDNA), which are composed of a phosphate backbone covalently bonded to four repeating nitrogenous bases: guanine (G), adenine (A), thymine (T), and cytosine (C). According to base pairing rules, guanine forms bonds only with cytosine and adenine forms bonds only with thymine. These base pairs of guanine-cytosine and adenine-thymine are held together by hydrogen bonds. When two strands of ssDNA contain corresponding bases in the correct order, they are called complementary. For self-assembly purposes, complementary ssDNA strands provide the spontaneous associative forces necessary to arrange building blocks together on the nanometer scale.⁶ On such a small scale, Brownian motion is a significant dissociative force; however, it is overcome by the strength of the base pair attractions, which act as a molecular Velcro between building blocks.

A major benefit of using ssDNA is that it can be synthetically manufactured to maintain complete control over the strength and type of its driving forces. The strength of the interactions between complementary strands is directly proportional to the number of corresponding base pairs in their sequences. The type refers to the programmable nature of DNA. Since the hybridization of complementary ssDNA sequences is the most

energetically favorable interaction, the joining of two building blocks is programmed by the functionalization of their surfaces with complimentary strands. The discovery of the Merrifield Synthesis in 1963 has since allowed for the manufacturing of ssDNA with specific nucleobase sequences and terminal functional groups, such as alkynes, thiols, and amines.⁷

Previous research has successfully produced self-assembling molecules that exhibit uniquely functionalized faces. Among these are Janus particles, which are spherical particles that contain two functionalized faces. These single-sided nanoparticles can be functionalized with two separate strands of ssDNA or amphipathic coatings and will self-assemble into organized features, such as micelles or layers. For example, spheres possessing one hydrophilic hemisphere and one hydrophobic hemisphere can form layers around other particles in aqueous solutions. Fig. 1 is an SEM image of a self-assembling coating composed of amphipathic Janus particles.

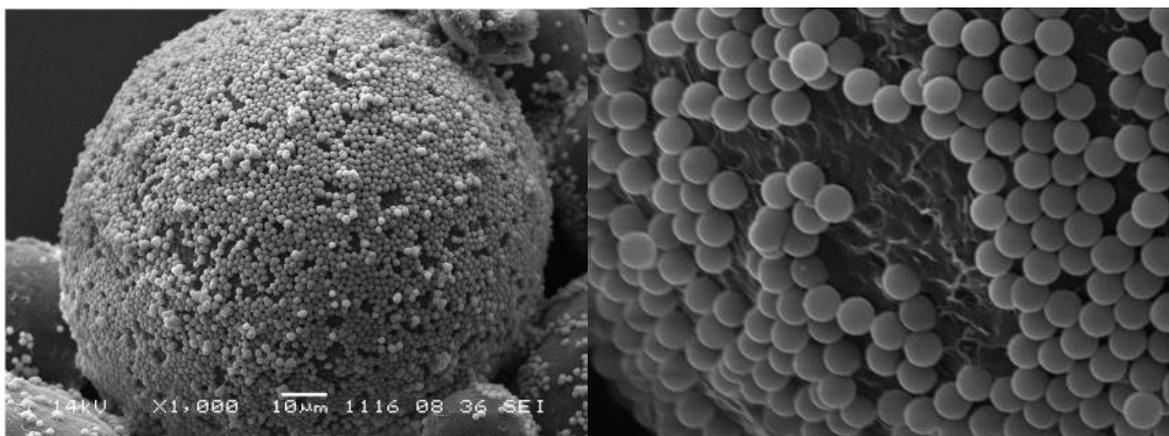


Figure 1. Self-assembly of a silica particle monolayer (amphipathic Janus particles) in aqueous solution around a wax ball.⁸

More recently, hydrogel cubes have been printed via photolithography, and assembled using DNA glue that promotes site-specific binding.⁹ The size of these cubes

ranges from millimeters to tens of microns. Despite these discoveries, neither of these methods has demonstrated the capacity to create three dimensional structures. The goal of this research project was to develop a method for creating self-assembling molecules that can be programmed to generate three-dimensional structures on the micrometer and nanometer scales. Fig. 2 shows a basic illustration of this method, including the formation of cubes and functionalization of specific cubes faces.

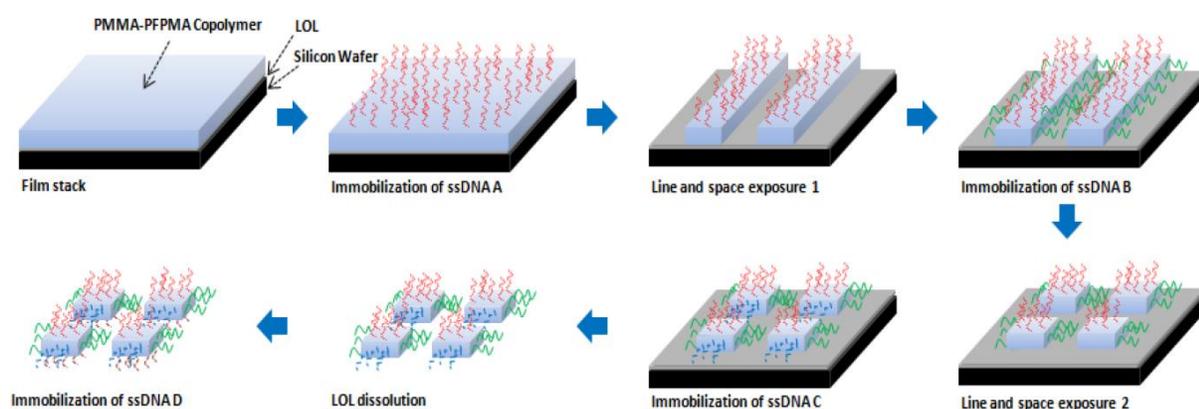


Figure 2. This process flow illustrates the use of photolithography to create cubes out of a patternable polymer using a line and space exposure pattern. The emerging features of these cubes are simultaneously functionalized (immobilized) with specific strands of DNA to create self-assembling blocks. A sacrificial lift-off layer is used to harvest the cubes.¹⁰

The ability of this method to produce cubes with six faces that can be individually functionalized with ssDNA would mark a breakthrough in self-assembly research. The applications of this method include smart packaging for drug capsules to ensure the targeted delivery of drugs to specific sites within the body, and the assembly of complex nanostructures for robotics and computing. The process steps of this method, which include biochemical and chemical engineering techniques, will be further outlined in detail.

Theory

Creation of Building Blocks

The building blocks are made using photolithography, which is a common industrial technique used in the production of microchips. This technique exposes patterns into photoreactive polymers, called photoresists, to create small features in underlying materials. Photoresists undergo intrinsic chemical reactions when exposed to certain wavelengths of light. These reactions change the solubility of the photoresist only within the exposed regions, allowing the exposed regions to be developed, or dissolved away. The selective dissolution photoresist region leaves behind defined patterns in the remaining photoresist material, and provides patterned access to the material below the photoresist. This patterned access allows one to etch the underlying material in that same pattern. Thus, this technique enables the creation of specific, three-dimensional features in an etchable material.

Photoresists are layered on top of these materials via spin coating. The material is supported by a substrate, typically a silicon wafer. Patterns are exposed into the photoresist from a light source located directly above the plane of the photoresist. The light passes through a mask, which blocks out light except for the desired pattern. Industrial processes employ masks to create complex circuit patterns for electronic devices; however, this self-assembly method only requires the simple patterning of an inert material into cubes. Fig. 3 outlines a basic photolithographic process from a cross-sectional view.

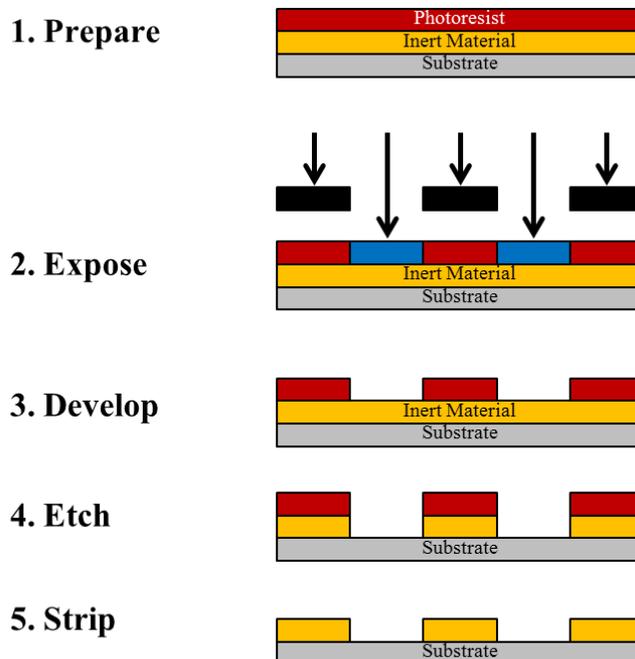


Figure 3. Cross-sectional view of photolithography as a means to create desired 3-D structures in an inert material. The final structures of the material match the pattern exposed into the photoresist.

The size of the building blocks is governed by the photolithographic process used to make them. The current limits of photolithography, as determined by the Rayleigh equation, allow for features as small as 40 nm.¹¹ For self-assembly applications, this limit was more than sufficient, as the majority of this research was carried out at the micrometer scale.

The building block polymeric material happened to be a photoresist itself; however its photochemical properties were not used for patterning. The specific photoresist used for the cubes was SU-8, an epoxy-based, negative photoresist. Whereas positive photoresists dissolve in their exposed regions, negative photoresists exhibit the opposite behavior. When exposed to 365 nm UV light, the epoxy groups in SU-8 cross-link, giving the polymer high chemical stability. The inert nature of the building blocks is derived from this chemical stability.

Functionalization of Cube Faces

The functionalization of cube faces with ssDNA has been the focus of this research project. The processes of photolithography and DNA conjugation are both well-understood; however, their compatibility with one another in an integrated method posed significant challenges. Three common DNA coupling chemistries were tested in order to identify the ideal chemistry to selectively bind ssDNA to faces of the inert building blocks. The most important requirement these chemistries was that it would allow for the successive, selective functionalization of cube faces. Without successive, selective functionalization, the programmable nature of DNA self-assembly is not possible. Other vital criteria for this chemistry included the following: high yield, high thermodynamic and kinetic stability, and a DNA-tolerant environment.

High yield, or a high conjugation of DNA, would ensure that the associative forces between cubes were great enough to overcome Brownian motion. The number of nucleobases in the complementary ssDNA sequences, as well as the surface density of ssDNA sequences immobilized on cubes surfaces, contributed to the strength of the associative forces. The bond between ssDNA and the cubes needed to be very stable so that the ssDNA could be permanently immobilized on the cube surfaces, and also so that the final, self-assembled structures could have applications in a wide variety of environments. A DNA-tolerant environment required that the functionalizing chemistry occurred in a neutral, low-temperature, aqueous environment, without any chemicals that would damage the DNA.

The three tested chemistries were carbodiimide coupling, thiol-ene click chemistry, and azide-alkyne click chemistry. The two functional groups contained in each of these

chemistries indicate combinations of the functional groups found on the cubes surfaces and ssDNA. These chemistries were tested using polymer microbeads instead of polymer cubes because the beads were ideal for basic quantitative analysis of the chemistries.

Carbodiimide Coupling

The first tested chemistry employed 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to mediate the conjugation of amine-functionalized ssDNA with carboxyl-functionalized polymer microspheres to form an amide linkage. EDC is a water-soluble, pH-sensitive carbodiimide that acts as a zero-length crosslinker between these two functional groups. Zero-length crosslinkers promote direct conjugation between two functional groups without adding additional atoms to the final bond. These characteristics are ideal for bioconjugation reactions. These reactions produced polymer microbeads functionalized with ssDNA. The ssDNA was then hybridized with its complimentary strand, which contained a fluorophore.

The use of fluorophores allowed the yield of this reaction to be quantitatively measured with a flow cytometer. The measured intensity of fluorescence corresponded to the amount of ssDNA bound to the surface of the microbeads. To test the stability of this amide linkage, the DNA-conjugated beads were subsequently reacted with a large molar excess of ethanolamine to render any remaining carboxyl group inactive to further DNA conjugation. A second ssDNA sequence containing a different colored fluorophore was reacted with the beads. No reaction was expected; however, fluorescence analysis indicated the presence of the second ssDNA sequence on the beads. Furthermore, the fluorescence of the first ssDNA

sequence decreased. These results suggested that the amide group was susceptible to being replaced by sequential reactions with other amine groups.

Fig. 4 contains graphs produced by the flow cytometer that show the occurrence of amide replacement. The x- and y-axes represent the intensity of green and red fluorescence, respectively. The left graph shows the intensity of beads that were functionalized with only green fluorescent ssDNA. The middle graph shows the intensity of beads that were functionalized with only red fluorescent ssDNA. The right graph shows the intensity of beads that were functionalized with green fluorescent DNA, followed by an identical reaction with red fluorescent DNA. There was a clear shift in fluorescence intensity from green to red.

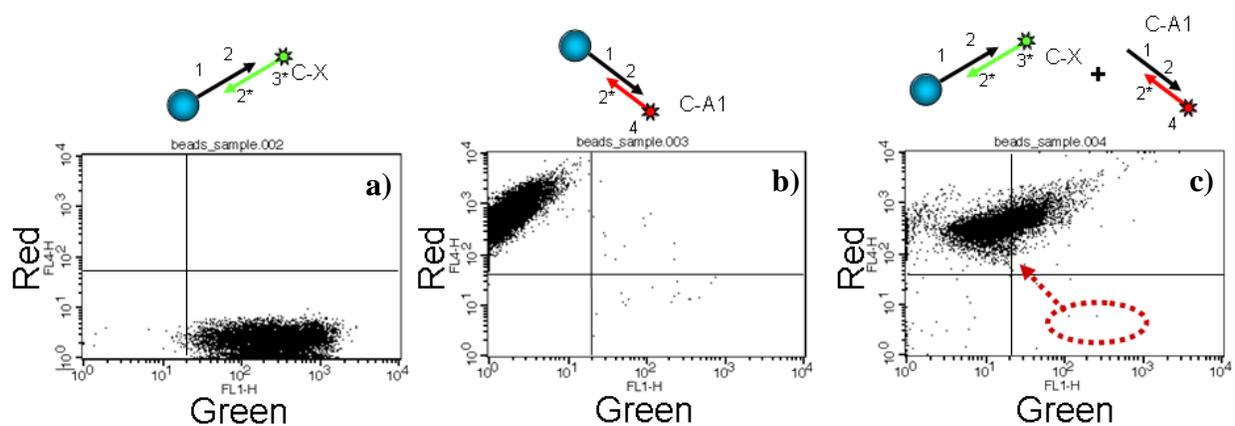


Figure 4. Flow cytometry data showing a) green fluorescent beads, b) red fluorescent beads, and c) a shift in intensity corresponding to the loss of the first ssDNA upon addition of the second ssDNA.¹²

Various control experiments were carried out to verify the validity of this data. To rule out fluorophore quenching as a possible source of error in this data, dual-functionalized ssDNA sequences bearing an amine group on one end and a fluorophore on the other end were used instead of the hybridized fluorophores. The same result was obtained. In addition, both sequences were mixed together with EDC to test if EDC facilitated DNA cross-linking. An electrophoresis analysis of this mixture indicated that the DNA did not cross-link. These

tests proved that DNA hybridization and EDC-facilitated cross-linking did not cause the apparent loss of DNA fluorescence. Thus, it was concluded that the covalent amide bond between the DNA and the bead surface was labile to further reaction with EDC.

Before ruling out the EDC-coupled amide linkage as a viable chemistry for this method, a Box Behnken design was created in order to optimize the selectivity of this chemistry. As discussed previously, this method relies on the selective functionalization of three-dimensional objects, the inert polymer cubes. This Box Behnken design tested many reaction conditions, the important ones being time, temperature, and pH. Fig. 5 shows the effect of these three conditions on reaction selectivity, measured as a ratio of green:red fluorescence. Higher selectivity is indicated by red, and lower selectivity is indicated in blue.

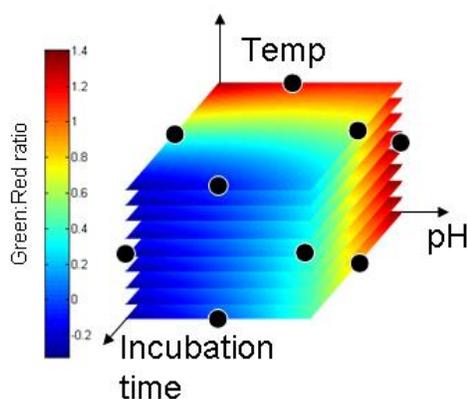


Figure 5. A Box Behnken heat map showing the effect of time, temperature, and pH on the selectivity of EDC-coupled amide formation.¹²

These design experiments found that selectivity increased with decreasing time, temperature, and pH. The optimal reaction conditions for highest selectivity were 24 °C, 30 minutes, and pH 7.5. Unfortunately, the maximum selectivity achieved under these conditions was 1.4, or about 60% green DNA and 40% red DNA on the same surface. The

minimum required selectivity for this method is 90%, so this chemistry was abandoned as a possible solution.

Thiol-ene Click Chemistry

Click chemistry is a class of chemical reactions that are efficient, high yielding, and tolerant of various solvents and functional groups. Among these reactions is the addition of a thiol group across the double bond of an alkene, referred to as thiol-ene click chemistry. This chemistry is a light-mediated radical reaction that can be activated at specific times and locations. The high levels of temporal and spatial control exhibited by thiol-ene click chemistry make it a powerful synthesis method.¹³ Several issues arose in trying to apply this chemistry to this method. First, the thiol-functionalized DNA exhibited nonspecific binding to the surface of the beads due to electrostatic interactions. These were minimized using Triton X-100, a nonionic surfactant. Second, the resulting cytometer data showed that the yield of this was extremely low. The results were consistent between combinations of thiol DNA/alkene beads and alkene DNA/thiol beads. This chemistry has not yet been ruled out, and the reaction conditions are currently being optimized to achieve a satisfactory yield.

Azide-alkyne Click Chemistry

Another member of the click chemistry group, azide-alkyne reactions are the most well-known click chemistries, and provide the same advantages to this method as thiol-ene click chemistry.¹⁴ The azide DNA/alkyne bead and alkyne DNA/azide bead reactions yielded high levels of DNA conjugation to the beads; however, both reactions failed to work when they were scaled up using actual polymer cubes. The alkyne photoresist cross-linked when

exposed, preventing the formation of cubes. This method required a positive photoresist, and the alkyne photoresist behaved as a negative photoresist. The azide functional group of the azide photoresist was destroyed when exposed to UV light for a short period of time. The azide group decomposed into nitrogen gas, which was observed as the formation of bubbles in the polymer. Since neither polymer was capable of being patterned into cubes, this chemistry was also abandoned.

At this point, it became clear that simple DNA conjugation chemistries were not directly compatible with the photolithographic process required to create the building block cubes. These three chemistries failed to solve this aspect of the method; thus, the method needed to be altered. While the click chemistries failed to provide a working reaction, the EDC-coupled amide reaction proved to be a successful route to functionalizing polymer surfaces with DNA, albeit a nonselective route. To take advantage of a working chemistry, but also ensure face selectivity, a protective photoresist layer was incorporated into the method to physically block reacted surfaces. This new method is similar to the process flow outlined in Fig. 2.

Hammer and Tongs Method

This new method was less elegant and contained many more steps compared to the original proposed method, hence the labelling of “hammer and tongs”. First, the LOL was spin-coated onto the wafer substrate. Next, the SU-8 layer was spun on above it, and cross-linked to become inert. The surface of this material was rendered reactive via brief O₂ etching, and silylated with 5,6-epoxyhexyltriethoxysilane to introduce a high surface density

of epoxide groups. Amine-functionalized DNA was then conjugated to the surface.

Instead of then exposing the inert polymer material to UV light and patterning it to expose new faces to be functionalized with different DNA, a protective layer of DNQ-Novolac photoresist was coated on top of the DNA layer. This photoresist layer was patterned to provide patterned access to the functionalized surface, as illustrated below in Fig. 6.

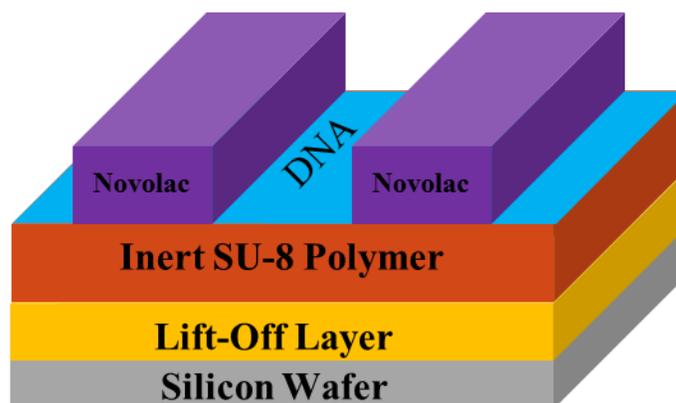


Figure 6. Illustration of the polymer stack including the LOL, SU-8 polymer with a top functionalized surface, and patterned DNQ-Novolac layer.

The original proposed that the inert SU-8 layer could be patterned into cubes via exposure and developing procedures; however, the inclusion of the protective photoresist layer required that the SU-8 be patterned by anisotropic O_2 reactive-ion etching (O_2 RIE). This procedure is a top-down technique that cuts trenches into the accessible regions down to the LOL. The post-etch polymer stack is illustrated in Fig. 7.

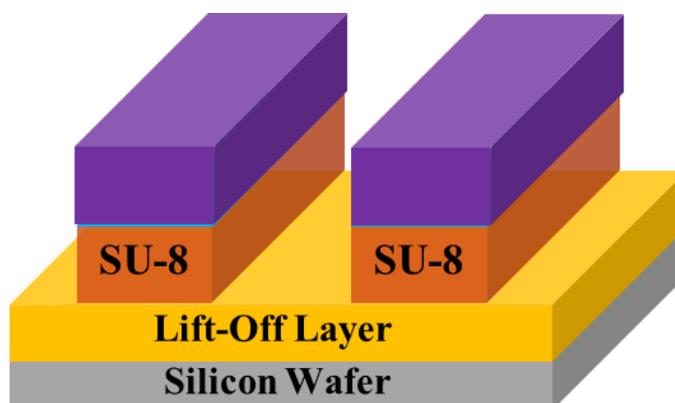


Figure 7. Illustration of post-etch polymer stack. Sections of the SU-8 were cut away to expose new sides of the material that will eventually be formed into many cubes.

These newly exposed faces could be rendered reactive with another brief O_2 etching, silylated with epoxide agent, and functionalized with a second sequence of ssDNA. During the second functionalization, the DNQ-Novolac layer would protect the previously functionalized surfaces. Repeating these process steps would create the desired self-assembling cubes.

Before this method was carried out in full, each step of the process was tested to confirm the compatibility of all of the steps in succession. Centimeter scale testing on allowed facile isolation of each process step to identify potential problems or optimizations for that step. In particular, the yield of the amine-epoxide reaction needed to be high enough to visualize the DNA conjugation, and the ssDNA needed to survive the application and removal of the DNQ-Novolac layer. The silylation reaction, ssDNA conjugation, O_2 RIE process, and LOL application were all optimized as well.

Process Flow

A clean, polished 100 mm silicon wafer was used as the substrate for this method. The LOL, polydimethylglutarimide (Microchem SF series), was spin-coated onto the wafer at 2500 RPM for 30 seconds, then baked at 250 °C for 5 minutes. Next, a solution of SU-8 (MicroChem SU-8 2002) dissolved in cyclopentanone was spin-coated on top of the LOL at 1000 RPM for 30 seconds, and then baked at 100 °C for 2 minutes. It was then exposed to near UV light (Dymax Bluewave® 200, 300-450 nm) for 10 seconds, and then baked a second time at 100 °C for another 2 minutes. The surface of the SU-8 layer was rendered reactive by O₂-RIE for a brief period of 20 seconds. The surface was then silylated with 5,6-epoxyhexyltriethoxysilane for 2 minutes, and then rinsed with deionized water.

The surface of the wafer was covered with 5 mL of pH 7.5 2-(N-morpholino)ethanesulfonic acid (MES). 50 µL of a 1 mM aqueous solution of amine-functionalized, fluorescent ssDNA were injected into the MES to conjugate the surface with DNA. The immobilization of DNA occurred overnight. The surface was then thoroughly rinsed with deionized water, and the presence of DNA confirmed using a Bio-Rad Molecular Imager plate reader to image the DNA fluorescence.

A layer of DNQ-Novolac (MicroChemicals AZ® 5214 E – IR) was then spin-coated on top of the DNA at 1500 RPM for 30 seconds, and baked at 100 °C for 10 seconds. The wafer was then taken to a mask aligner, and exposed to near UV light through a mask for 15 seconds. Fig. 8 displays examples of mask patterns used to create cubes. Line and space patterns allow for multiple DNA conjugations of single cubes. By rotating the mask 90°, superimposed line and space patterns form squares.

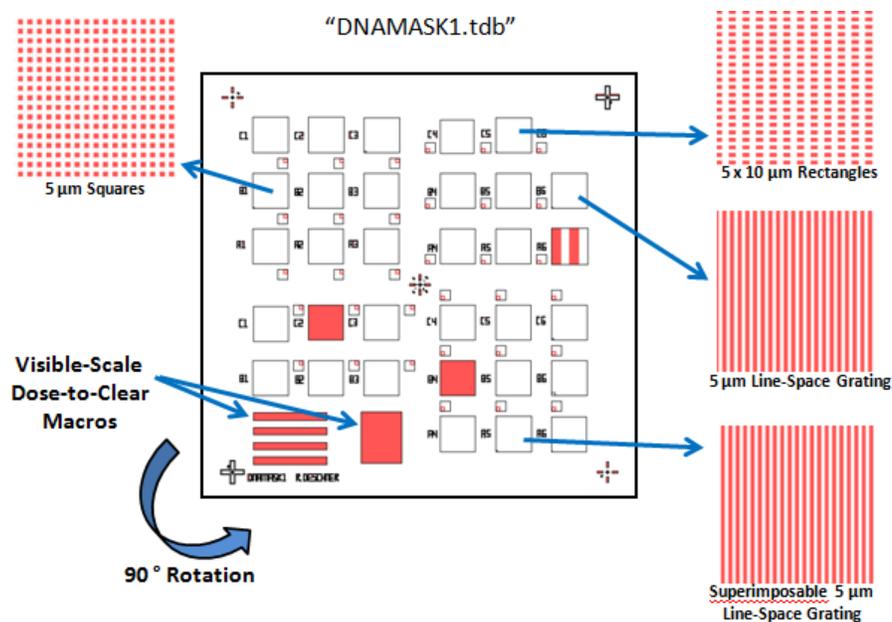


Figure 8. A layout of a chromium-on-quartz photomask. Various domains, including square, rectangular, and line and space patterns can be created by perpendicularly superimposed exposures.¹⁰

The exposed wafer was then submerged in a bath of 2.38 wt% TMAH for 30 seconds to develop the mask patterns. A 15 minute O₂-RIE process etches away the exposed SU-8 regions to create three dimensional structures, as illustrated in Fig. 7. The desired square domains were physically isolated from the rest of the wafer using a diamond cutter. The isolated domains were lifted-off in a 1 mL solution of Clariant potassium tetraborate and suspended in solution.

This process was repeated for the complimentary ssDNA sequence. The solutions of complimentary cubes were combined with aliquots of magnesium sulfate and analyzed using optical microscopy.

Results

After completing the individual testing of each step, the method was implemented with two ssDNA functionalization steps on a centimeter scale. More functionalization steps could have been implemented; however, no new significant data would have been obtained because the same steps would have been repeated. This method was applied to a wafer shard, upon which the LOL, SU-8 layer, and DNQ-Novolac layer were coated. The top surface of the SU-8 layer was functionalized with ssDNA containing a fluorescein fluorophore. Half of the shard was covered with a mask, and the other side was exposed and developed to remove the DNQ-Novolac layer. The etched, newly-exposed SU-8 surface was then functionalized with ssDNA containing a Cy5 fluorophore. The shard was then imaged using a Bio-Rad Molecular Imager plate reader. The resulting picture, Fig. 9, demonstrated the potential of the Hammer and Tongs method to selectively functionalize polymer surfaces with specific sequences of ssDNA to create self-assembling cubes. It also proved the tolerance of DNA with the DNQ-Novolac coating.

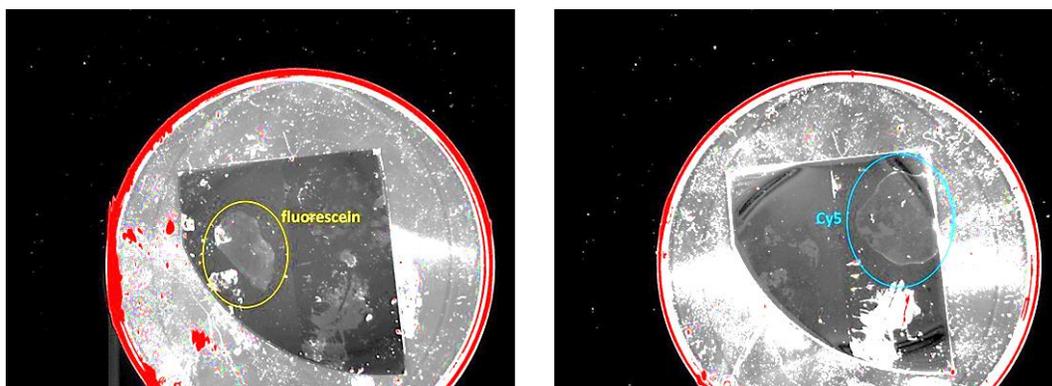


Figure 9. These images of the same wafer were taken using different filters to see the different fluorophores. There are two clear patches of ssDNA that were selectively functionalized of different sides of the wafer.

This method was repeated for two overlapping DNA conjugations to further highlight the potential for selective functionalization. Half of the first DNA patch was covered with the DNQ-Novolac layer, and the other half was exposed, etched, and reacted with a second fluorescent ssDNA sequence. The protective layer was completely removed, and the fluorescence of both patches was observed, as shown in Fig. 10 below.

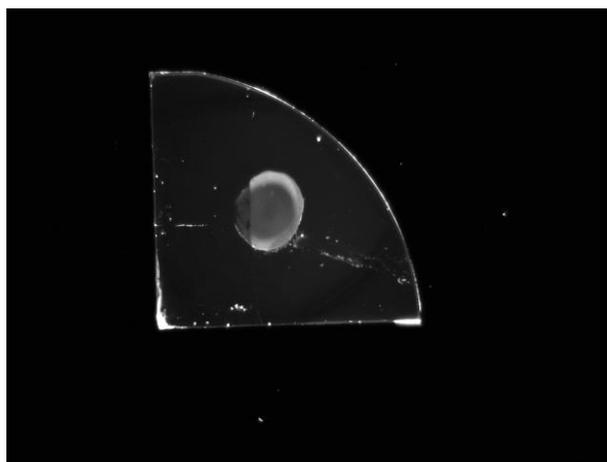


Figure 10. Proof of selective DNA functionalization on overlapping fluorescent regions. The vertical line indicates the border between DNA strands.

Given this successful result, the method was scaled up further. Instead of selectively functionalizing two regions of the same two-dimensional surface, the process was applied to three dimensional structures as illustrated by Fig. 6 and Fig. 7. The Hammer and Tongs method was applied to two wafers, each functionalized with a complementary strand of ssDNA. Only one face was functionalized for the sake of clear data analysis. Doing so allowed for the clear identification of cube dimers versus single cubes. The Hammer and Tongs method successfully produced self-assembling cubes that were imaged using an optical microscope, as shown in Fig. 11. This image displays a section of aqueous solution containing single cubes, as well as vertically and horizontally aligned cube dimers.

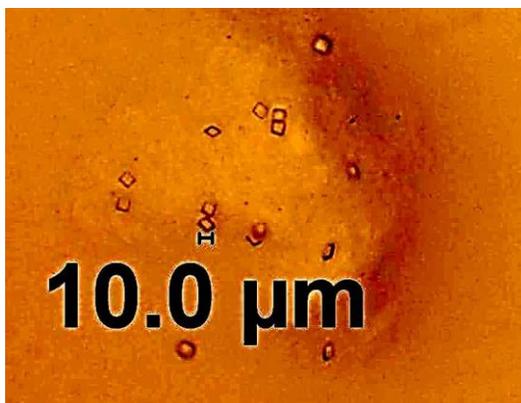


Figure 11. Image of self-assembled cube dimers suspended in an aqueous solution.

This result proves the capability of the Hammer and Tongs method to create programmable, three-dimensional, self-assembling structures on the micrometer scale. Looking forward, the optimization of the self-assembling process for better data analysis will be crucial in further developments of this method. In addition, the multi-functionalization of cube faces to create more complex structures will demonstrate the full potential of this method for three-dimensional self-assembly.

Conclusion

The development of the successful Hammer and Tongs method marks a breakthrough in self-assembly research. Complex, three-dimensional structures on the micro- and nanometer scale can be created via programmable self-assembly. Compatibility between DNA conjugation chemistries and photolithographic patterning of polymers that allows for selective functionalization has not yet been discovered; however, employing a second photoresist as a protective layer provides a viable, alternate route. Despite introducing many new steps to the original process, this method successfully facilitated the programmed assembly of cube dimers. At the time of this paper, this method is being applied to the formation of more complex structures. A significant improvement to the efficiency and elegance of this method would be the discovery of a compatible chemistry between photolithography and DNA conjugation.

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