

Nanopatterning of Recombinant Proteins Using Block Copolymer Templates

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ABSTRACT: Exploiting the microphase separation of block copolymers is a facile method of synthesizing nanometer-sized structures. The interactions of recombinant proteins with block copolymer surfaces displaying nanoscale order could be used to investigate intercellular signaling and for creating arrays of nanoreactors for lab-on-a-chip applications. A block copolymer of norbornene and norbornenedicarboxylic acid (NOR/NORCOOH) was synthesized and its hydrophilic block loaded with metal ions. Protein binding ability was examined by exposing the metal-loaded copolymer film to histidine-tagged green fluorescent protein (hisGFP), washing with detergent to remove nonspecifically bound protein, and measuring the surface fluorescence. Results showed that nickel ions on the copolymer surface had superior hisGFP binding ability to copper and iron. Further investigation comparing the binding of hisGFP and GFP lacking the histidine tag showed that the nickel templated block copolymer was binding only hisGFP. It was concluded that the chelation between nickel on the polymer surface and histidine groups on the protein is the only significant binding force in the hisGFP–copolymer system.

Introduction

The ability to immobilize proteins to nanometer-sized patterns has become a major challenge for the development of bioengineered surfaces. Nanopatterned surfaces are known to influence cell function through surface-triggered interactions.¹ The ability to vary the topology and separation between nanopatterned recombinant proteins on the surface of a block copolymer may lead to a better understanding of cellular signaling. In addition, the ability to spatially control the immobilization of small amounts of recombinant proteins may provide better platforms for the study of single molecular events, such as through AFM force measurements.

This study examines the ability of the diblock copolymer surface to selectively bind hisGFP. Kumar and Hahn demonstrated the feasibility of nonspecific protein immobilization on block copolymer patterns in a recently published study.² To accomplish specific protein adsorption to a block copolymer surface, we synthesized a norbornene block copolymer and loaded different metal ions to determine the most effective ion to use in protein binding. Copper,³ iron,⁴ and nickel⁵ ions have been mentioned in the literature as being effective in chelating histidine-tagged proteins. Currently, nickel chelation columns are used regularly to separate histidine-tagged proteins from a mixture in order to isolate the tagged protein⁶ in a process first introduced by Porath et al.⁷

Monomers of norbornene and norbornenedicarboxylic acid were copolymerized together to produce an amphiphilic diblock copolymer. The hydrophilic block of these copolymers was used to template the formation of metal nanoparticles. Robert Cohen developed a method where the carboxylic acid block of a diblock copolymer was used to template the formation of nickel

nanoparticles within the hydrophilic blocks of these copolymers.^{8,9} While this metal-templating technique has been successful in the methyltetradecene–norbornenedicarboxylic acid system, the same principle holds for the polymer systems used in this experiment and was verified in this study through electron microscopy. Ring-opening metathesis polymerization (ROMP) was chosen to form the norbornene–norbornenedicarboxylic acid (NOR/NORCOOH) diblock copolymer.^{10–13}

The protein species used is a modified version of green fluorescent protein (GFP). A hexahistidine (his₆) affinity tag is grafted to the N' terminus of the GFP through the expression of recombinantly modified DNA.^{14,15} This histidine tag has a strong affinity for metal ions, with which it forms stable complexes.¹⁶ Studies have shown that the histidine tag does not affect the structure and function of GFP, so that the standard methods of analyzing for GFP can also be used with hisGFP. Binding of the hisGFP to the surface can thus be connected to the detection and intensity of fluorescence coming from a film exposed to hisGFP.

Once the nickel ion was selected from the three metals being tested, the actual method of protein binding was investigated. Specifically, it must be known whether it is the his₆ tag on the protein that is responsible for binding or whether other functional groups present on the protein can interact with and bind to the metal groups on the copolymer surface. To separate these two effects, the binding of hisGFP was compared directly to that of untagged GFP. In addition, it must be established the metal nanoclusters in the copolymer are responsible for binding hisGFP. Therefore, the experiment must also eliminate the possibility that the norbornene copolymer itself has an affinity to the protein or to the his₆ tag.

With increasing interest in microfluidic devices and the idea of lab-on-a-chip technology, this ordered polymer surface may aid in the production of arrays of single nanoreactors in microfluidic devices. This could function in applications including combinatorial chemistry for drug discovery and high throughput analysis in genomics and proteomics. Nanoscale confinement studies depend on the development of appropriate nanoscale

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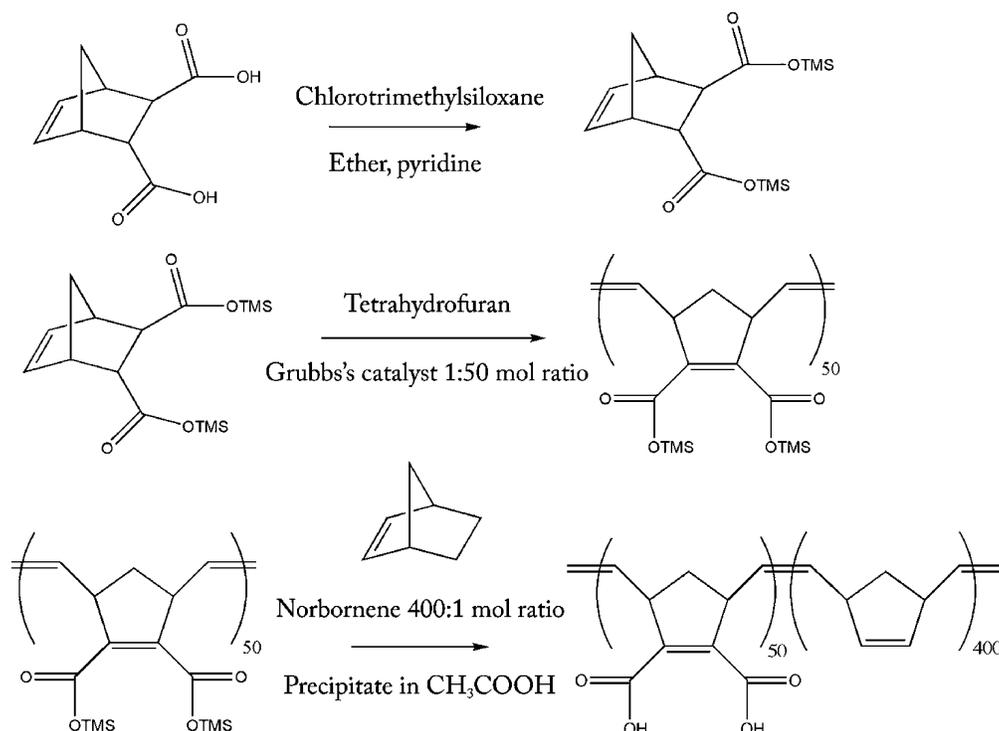


Figure 1. ROMP synthesis of NOR₄₀₀NORCOOH₅₀, starting with the protection of acid group with TMS, formation of the NORCOOTMS homopolymer, and block copolymerization of NOR onto the living NORCOOTMS homopolymer.

patterns such as those present by the self-assembly of block copolymers. The polymer surface, functioning as a hexahistidine (his₆)-tagged protein chelator, could serve as the basis for a fast protein separation device in the process of protein purification.

Experimental Section

Green fluorescent protein (GFP) was purchased from ClonTech Laboratories, as part of the Living Colors recombinant protein line (rGFP). hisGFP was synthesized through the expression of recombinantly modified DNA and purified according to literature procedures.¹⁵ Tetrahydrofuran (THF) and norbornene (NOR) were purchased from Aldrich, distilled over sodium, and then degassed three times through a freeze/pump/thaw process. 5-Norbornene-2-endo,3-exo-dicarboxylic acid (NORCOOH) was purchased from Aldrich, dried in a vacuum, and stored in an argon-filled MBraun LabMaster100 glovebox. Grubbs' second-generation ROMP initiator [1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene)(tricyclohexylphosphine)ruthenium] was also purchased from Aldrich, stored in the glovebox, and used as received. All reactants were distilled and dried before use, and the polymerization took place in the glovebox. Fluorescence testing was performed using a Perkin-Elmer LS55 luminescence spectrometer with excitation at 395 nm and luminescence at 508 nm.¹⁷ Data were taken by averaging a series of 10 readings into one fluorescence data value. Three films of each type were tested in order to establish experimental error and account for variations in luminescent intensity across the film surface.

Synthesis of Protected NORCOOTMS Monomer. 10 g of NORCOOH was dissolved in 400 mL of dry ethyl ether and allowed to stir for 4 h. With the NORCOOH completely dissolved, 10 mL of pyridine and 5 mL of chlorotrimethylsiloxane were added to the stirring solution in the glovebox. Pyridine hydrochloride precipitated from the stirring ether, and this white precipitate was filtered out by pouring the reaction mixture through a 1 cm bed of Celite. The product, norbornenedicarboxylic trimethylsilyl ester (NORCOOTMS), was recovered and purified through three cycles of recrystallization in ethyl ether. The final, pure NORCOOTMS appeared as a white powder and was vacuum-dried and stored in the glovebox.

Synthesis of NOR₄₀₀NORCOOH₅₀ Diblock Copolymer. The diblock copolymer was formed by first initiating the polymerization

of the NORCOOH block. NORCOOTMS was dissolved in THF to a concentration of 1 mmol/mL. Grubbs' second-generation initiator was added so that the mole ratio between initiator and NORCOOTMS was 1:50, giving a dark purple solution. The purple solution was vigorously stirred and allowed to polymerize for 24 h. At the end of this period, the purified and dried NOR was added to the reaction mixture. NOR was dissolved in solution in THF at 0.2 mmol/mL. This reaction was allowed to continue for 6 h, and then it was removed from the glovebox and halted with the addition of 200 μ L of ethyl vinyl ether. The polymer was recovered by precipitation in a chilled mixture of 400 mL of methanol, 40 mL of distilled water, and 5 mL of glacial acetic acid. The solid polymer obtained from this process was white with a slight purple tint caused by trace amounts of the Grubbs' catalyst and was washed thoroughly with pentane to remove unreacted monomer. The final washed product was dried in a vacuum and then redissolved in pure THF at 1 mg/mL. This mixture was used to form NOR/NORCOOH films. A schematic of this reaction process is shown in Figure 1.

Films used for TEM study were cast from a solution of THF and 1% w/v nickel nitrate in 5 cm diameter, flat-bottomed containers made from Bytac PTFE-coated aluminum sheets within a THF-filled desiccator over the course of 7 days. Upon casting, the polymer films were microtomed and examined in the TEM. Films that were used for surface binding tests were cast on the surface of glass slides that had been stored at 120 °C and sterilized with alcohol and acetone. 3 mL of the polymer solution was spread evenly over the slide, forming a uniform, clear film over the slide. To prepare the NOR/NORCOOH block copolymer for protein adsorption, films of the copolymer were cast on glass slides which had been heated to 120 °C and then washed in acetone and ethanol. Films were cast by allowing 3 mL of polymer solution (at 1 mg/mL) to evaporate on the slide, giving a uniform thin film of NOR/NORCOOH on the slide. In all experiments, the proteins hisGFP and GFP were dissolved in aqueous solutions at a concentration of 1 mg/mL.

Binding Study of hisGFP on NOR/NORCOOH Surface with Different Metal Ions. To test the hisGFP binding effectiveness of different ions, three metal ion species were examined: nickel, copper, and iron. NOR/NORCOOH films were soaked in solutions of metal salts to add metal ions to the surface -COOH groups. Nickel nitrate (Ni(NO₃)₂), copper sulfate hexahydrate (CuSO₄·6H₂O),

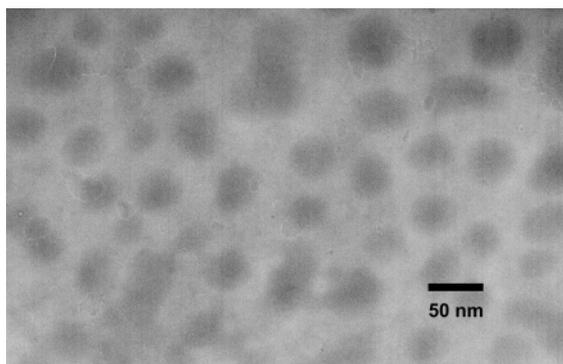


Figure 2. TEM micrograph of the nickel-loaded NOR₄₀₀NORCOOH₅₀ diblock copolymer.

and iron chloride (FeCl₃) were dissolved in water at a concentration of 100 μ g/mL. NOR/NORCOOH films were exposed to the aqueous solution for 12 h. Three NOR/NORCOOH films were exposed to each metal type to provide experimental spread during data collection. The films were removed from the metal solution, washed with deionized water, allowed to dry for 1 h, and then exposed directly to the hisGFP solution. hisGFP exposure was limited to 10 min, after which the films were washed with 1% Tween 20 detergent solution. This step was performed in order to remove all proteins not specifically bound to the metal groups on the surface of the polymer. The treated films were immediately tested in a fluorescence spectrometer to quantify the amount of protein held on the polymer surface. Baseline was established by taking the fluorescence of the empty test chamber as well as that of the blank glass slide and the polymer both with and without metal loading. Since these values were very similar, they were averaged together to give a single baseline value to which fluorescence of bound hisGFP was compared.

Fluorescence of hisGFP on NOR/NORCOOH. Polymer films were cast and loaded with nickel ions in an identical fashion as the

previous metal ion–hisGFP affinity test. Two identical film sets were cast to test the hisGFP binding directly against that of GFP without the histidine tag. This meant casting two sets of nickel-loaded block copolymer as well as two sets of plain, non-metal-containing copolymer. Two glass slides were also used, as before, to ensure the glass gives no fluorescent background readings. These test films were exposed directly to the hisGFP or GFP solution. Protein exposure was limited to 10 min, after which the films were washed with 1% Tween 20 detergent solution. This step was performed to remove all proteins not specifically bound to the metal groups on the surface of the polymer. The treated films were immediately tested in a fluorescence spectrometer to quantify the amount of protein held on the polymer surface. Baseline was established by taking the fluorescence of the empty test chamber as well as that of the blank glass slide and the block copolymer both with and without metal loading. As in the previous experiment, the empty chamber, glass slide, and polymer with and without metal gave nearly the same fluorescence value and so were averaged together to give a single baseline value. Because there were no competing fluorescence sources in the system, it was possible to detect bound GFP fluorescence and bound hisGFP fluorescence in order to compare binding of the GFP against hisGFP.

Results and Discussion

Gel permeation chromatography (GPC) was used to examine the molecular weight and polydispersity of the NOR/NORCOOH diblock copolymer produced from ROMP. It was found that the weight-average molecular weight of the copolymer was within 5% of the target (46 773 g/mol) for a 400/50 block ratio, with a polydispersity index (PDI) of 1.21. Transmission electron microscopy (TEM) allowed direct observation of the copolymer's microphase-separated morphology. The 400/50 NOR/NORCOOH block ratio produced spherical domains of NORCOOH with an average diameter of 30 ± 5 nm, which can be seen in Figure 2.

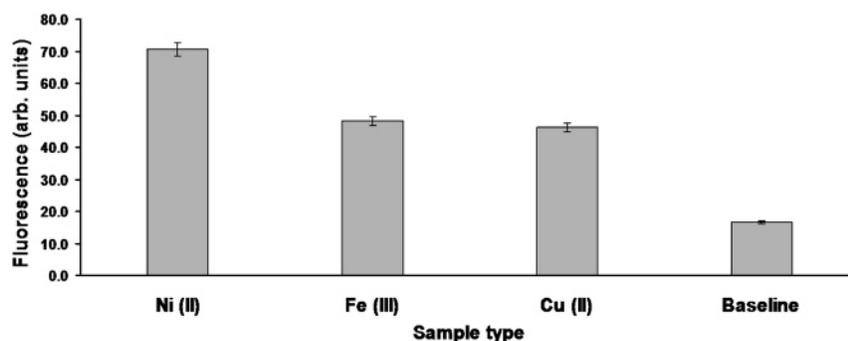


Figure 3. Fluorescence data obtained from block copolymer surfaces loaded with different metal ions and exposed to hisGFP. The nickel-loaded block copolymer exhibited significantly higher affinity to his GFP than other metal-loaded polymer samples.

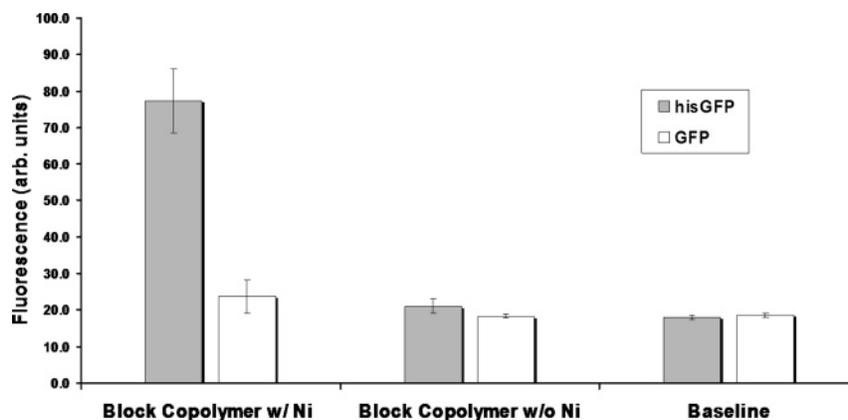


Figure 4. Comparison of hisGFP vs GFP fluorescence on various surfaces. Only the fluorescence caused by hisGFP binding on the nickel-loaded block copolymer surface was significantly above baseline.

The glass slide and polymer films that were not exposed to hisGFP achieved only baseline fluorescence, as expected. The metal-loaded films, on the other hand, showed significant fluorescence. Among the three metal ions tested in the block copolymer, nickel proved the most effective, giving a fluorescence of 70.58 ± 2.11 arbitrary intensity units. Copolymer films treated with copper and iron ions also showed hisGFP binding but showed significantly less fluorescence, as seen in Figure 3. Copper-treated films showed fluorescence of 48.32 ± 1.45 , and iron was nearly the same value at 47.35 ± 1.40 . The results in Figure 3 confirm that there is metal ion binding activity at the surface of the copolymer and that nickel is the most effective ion in binding hisGFP.

While the first experiment was designed to identify the most effective metal ion in binding the recombinant protein, the second test was designed to ensure that binding of the hisGFP was due only to the his₆ affinity tag on the protein N' terminus. Therefore, hisGFP and GFP without the his₆ affinity tag were tested against each other on identical films to show the importance of the his₆ tag to the copolymer surface binding in this system.

A baseline fluorescence value was established as in the previous test, and it was found that all experimental controls gave very similar fluorescence readings, which were averaged together and treated as the baseline, as seen in Figure 4. Data for the hisGFP and GFP on the nonmetal loaded polymer were analyzed with ANOVA, which showed them to be statistically close to baseline, suggesting that the polymer with no metal had little or no affinity for either protein. ANOVA also showed that the nickel-loaded copolymer surface held a significant amount of the hisGFP with a detected fluorescence of 77.43 ± 8.74 . The nickel-loaded polymer could not significantly bind the untagged GFP, as its fluorescent intensity was not significantly above baseline.

These data suggest strongly that the interaction between the hisGFP and the metal at the polymer surface is significantly higher than any other nonspecific interactions in the system and that the metal-loaded surface is indeed specifically interacting with the his₆ affinity tag.

Conclusions

The experiments performed on this block copolymer system show that nickel is the most effective metal in chelating histidine-tagged green fluorescent protein and that the metal-

loaded surface of the norbornene block copolymer is capable of binding histidine-tagged green fluorescent protein while being unable to bind green fluorescent protein without histidine tags. While there are undoubtedly nonspecific interactions like hydrogen bonding and Coulomb attraction between the polymer, metal, and the target protein, it appears that only the histidine metal chelated protein can remain through the process of washing performed in these experiments. Future work upon this project will focus on studying the composition of the metal-loaded copolymer surface and the actual location of these histidine-tagged proteins on the copolymer surface.

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