7 Conclusions and Future Work

7.1 Summary of Research

The main focus of this research has been on the development of a new cloning strategy that can be easily adapted for the biosynthetic production of modular, sequentially-modifiable, surface-active PAA's. To accomplish this objective, the following specific aims were proposed in Chapter 1:

Specific Aim #1: ***Design a PAA sequence that will self-assemble on aluminum oxide surfaces to form brush layers, and produce this PAA in genetically engineered E. coli***

Specific Aim #2: ***Develop a new cloning strategy to meet the need for a simple and universal method for constructing the genes required for producing specifically designed, high molecular weight brush-forming PAA's in genetically engineered E. coli***

The accomplishment of these goals has been presented throughout this work. Specific Aim #1 was completed by demonstrating the feasibility of producing two unique PAA diblocks, each consisting of a glutamate-based anchor block and either a proline-based or zwitterionic tail block. Specific Aim #2 was completed through the development of a viable new cloning strategy and its use to design and produce a specifically defined, high molecular weight PAA diblock.

Prior to this research, there did not exist a cloning strategy that was sufficient for the streamlined production of specifically designed, high-molecular weight brush-forming PAA's. Therefore, the results presented have the potential to significantly increase the ease in which we and other investigators will be able to produce surface-active PAA’s for a wide variety of fundamental studies and practical applications. The following sections will summarize the conclusions drawn from each chapter, as well as the proposal of additional experiments and alternative applications that may be investigated.

7.1.1 Conclusions from Chapter 3

In Chapter 3, the feasibility of producing and isolating a unique PAA that was designed to form brush layers on alumina surfaces was demonstrated. Based on prior work, it was determined that our initial brush-forming PAA design should consist of a poly-glutamate anchor block and poly-proline tail block. Using conventional recombinant DNA techniques, a gene encoding for a PAA diblock with the composition $Pro_{39}Glu_{10}$ (20mol% anchor) was constructed and cloned into a genetically engineered strain of *E. coli*, where it is expressed as a fusion protein. As stated, we were unable to attain the composition required for optimum brush extension (5%mol anchor) using these cloning methods.[1, 2] However, we deemed it worthy to carry through with the production of this PAA design. After purification using a series of chromatographic steps, the fusion tag was removed and the PAA product was isolated and its composition was confirmed.

Unfortunately, we were unable to produce sufficient amounts of a pure Pro_mGlu_n construct for surface adsorption studies to characterize its brush-forming ability on alumina surfaces. This was due to limited production and purification facilities. However, the successful biosynthetic production and isolation of the $Pro_{39}Glu_{10}$ product is a significant accomplishment since it is the first biosynthetically produced PAA diblock specifically designed for brush-forming applications. In addition, this work led to the successful demonstration of the brush-forming ability of the fusion product (Chapter 4) as well as demonstrated the need for an alternative cloning method for the streamlined production of optimized brush-forming PAA's (Chapter 5).

7.1.2 Conclusions from Chapter 4

In Chapter 4, the successful brush-forming ability of a unique PAA consisting of a poly-glutamate anchor block, a poly-proline linker region and a ‘capping domain’ was demonstrated. During these experiments, the fusion product produced and isolated in Chapter 3 (Trx-$Pro_{39}Glu_{10}$) was used. Here, the His-Patch Thioredoxin was a convenient model for a capping domain since it possessed several desirable characteristics: a globular, three-dimensional structure; hydrophilicity; and the fact that it is relatively uncharged at the experimental conditions (pH 6-7).

Dr. William A. Ducker's research group conducted the necessary surface adsorption studies for this work. Ellipsometry measurements confirmed that the PAA product absorbed onto a positively charged alumina surface from an aqueous solution at pH 6-7 with a density of 3-7 mg/m^2. Atomic force microscopy measurements also confirmed that the adsorbed PAA generated a steric force sufficient for colloidal stabilization. The brush layer thickness was determined to be ~15nm, which was consistent with the dimensions of the PAA obtained with dynamic light scattering measurements conducted by Dr. Richey M. Davis' research group. These results confirmed the feasibility of using biosynthetically produced PAA's for brush-forming applications. In addition, this work indicated that the potential may exist to design a unique brush-forming PAA that includes a biologically active protein as the capping domain.

7.1.3 Conclusions from Chapter 5

It was concluded from the work in Chapter 3 that an alternative approach was necessary to attain the goal of producing high molecular weight surface-active PAA's. Conventional recombinant DNA techniques, as well as existing cloning strategies located in the literature for the production of synthetic structural protein polymers, were not sufficient for the construction of the long genetic sequences necessary for producing optimized brush-forming PAA's of sufficient molecular weight and composition.

In Chapter 5, we introduced a universal cloning strategy for the facile production of highly specified high molecular weight PAA's. The strategy incorporated the following desirable features: modular gene construction, sequentially-modifiable, minimal amino acid requirement, and versatility. The sequentially-modifiable nature of the strategy allowed us to insert multiple DNA modules ~80 bp in length, thus constructing a ~240 bp sequence encoding for a ~80-mer PAA tail block. The only amino acid requirement was the incorporation of a glycine residue at the C-terminus of each PAA module. These glycines have a minimal effect on the PAA design, and may actually enhance the flexibility of the tail PAA block, thus promoting better tail extension and brush formation. The method is versatile due to the fact that it is compatible with commercially available expression vectors for a variety of different systems (prokaryotic, yeast and

mammalian) and only uses basic molecular biology and recombinant protein production methods.

The successful completion of the preliminary experiments confirmed that long genetic sequences could be easily constructed, resulting in a level of control over PAA composition and molecular weight that is unmatched by current polymer synthesis methods. Therefore, the work presented in this chapter successfully confirmed the feasibility of the new cloning strategy.

7.1.4 Conclusions from Chapter 6

In Chapter 6, the feasibility of applying the cloning strategy introduced in Chapter 5 to the production of a unique PAA diblock designed to form brush layers on aluminum oxide surfaces was demonstrated. Similar to the diblock produced in Chapter 3, this PAA construct contained a 10-mer glutamate-based anchor block. Due to the modular nature of the new cloning strategy, a long genetic sequence encoding for a 100-mer zwitterionic PAA tail block was constructed. The zwitterionic PAA sequence was designed to have a zero net charge at pH~7. It was hypothesized that such a PAA sequence would not adsorb on charged surfaces, thus enabling it to act as a universal tail block for metal oxide systems. We were successfully able to demonstrate the effectiveness of applying the new cloning strategy to the construction of the DNA sequences encoding for PAA's designed to form optimized brush layers (~8mol% anchor), where composition and molecular weight were controlled at the monomer level. Once the synthetic gene was constructed, it was expressed as a fusion protein within genetically engineered *E coli.* After purification using a series of chromatographic steps, the fusion tag was removed and the PAA product, referred to as T_4A, was partially isolated and its composition was successfully confirmed.

Unfortunately, we were unable to produce sufficient amounts of a pure T_4A construct for surface adsorption studies to characterize its brush-forming ability on alumina surfaces. As stated, during our experiments it became evident that the zwitterionic PAA block of the product may be inherently unstable. It is hypothesized that the zwitterionic PAA sequence is susceptible to proteases, which can be avoided by the addition of protease inhibitors in all buffers. However, the exact cause of the instability

could not be determined at this time, due to limitations in the ability to scale-up PAA production for additional studies.

This is an unfortunate complication that jeopardizes the practicality of this PAA design. Obviously, if unstable, this PAA would not be suitable for brush-forming applications. However, the successful biosynthetic production and partial isolation of the T_4A product can be viewed as a significant accomplishment. We have shown that we have developed an effective strategy for the design, cloning, expression and purification of specifically designed, high molecular weight brush-forming PAA's. The methodology presented, therefore, can be universally applied towards many other PAA designs and applications, paving the way for breakthroughs in fundamental material science research studying self-assembly phenomena.

7.2 Future Work

The results of this work provide the opportunity for future experiments. These experiments may continue to pursue the long term goals of the presented work or investigate other applications where the technology developed here can be applied. Each of these scenarios is discusses below.

7.2.1 Continuation of Current Project

Through the demonstration of the feasibility of using recombinant DNA methods to specifically design and biosynthetically produce unique brush-forming PAA's, the objective of this work has been achieved. However, one of the long-term goals of this research was to synthesize new and unique brush-forming PAA's for the steric stabilization of colloidal alumina suspensions. To accomplish this task, the following future experiments must be conducted:

1) Scale-up production of $Pro_{39}Glu_{10}$

This first task must be accomplished in order to provide sufficient PAA material for investigating the solution and surface adsorption characteristics of the construct. The work of this project focused solely on demonstrating the feasibility of designing and producing unique brush-forming PAA's. Due to the lack of proper facilities, sufficient

scale-up of production and purification of this PAA was not possible at the time of this project. In the future, access to larger bioreactors and chromatographic systems will allow for the relatively straight-forward scale-up of the unit operations used during the production and purification steps described in this work.

2) Solution and surface adsorption studies of $Pro_{39}Glu_{10}$

Once sufficient PAA material is obtained, the solution and surface adsorption characteristics of each diblock can be investigated. The methods used here will be similar to those applied in Chapter 4 during the investigation of the brush forming behavior of the Trx-$Pro_{39}Glu_{10}$ construct. These methods include: dynamic light scattering, ellipsometry and atomic force microscopy (AFM). Ellipsometry will be used to verify that the PAA adsorbs onto alumina surfaces and measure the adsorbed amount. AFM will be used to determine the forces present between alumina surfaces coated with adsorbed PAA and the length scale of the resulting brush layer. Together, these experiments will help determine if the unique PAA's produced will adsorb onto alumina surfaces and self-assemble to form brush layers that are sufficient for steric stabilization.

3) Scale-up production of T_4A for stability studies

We could not determine the cause of the apparent instability of the zwitterionic PAA block due to insufficient material for additional experiments for the same reasons given above. For example, with more material, experiments can be planned to investigate if the zwitterionic block is susceptible to proteases. This can be studied by observing how the addition of protease inhibitors in all buffers used during PAA purification affects stability. If the instability can be avoided, this PAA design may still have promise. However, if the stability can not be controlled, unfortunately, this PAA would not be suitable for brush-forming applications.

4) Design and production of additional PAA diblocks

By applying the methods developed in this research, the design of additional PAA diblocks should be relatively straightforward. For example, combining the concepts of the two diblocks produced in this work may result in an improved PAA design. Such a

diblock would consist of a short, glutamate-based anchor block with a long, proline-based tail block. However, by applying the new cloning strategy, a much longer tail block could be constructed, improving upon the $Pro_{39}Glu_{10}$ design.

7.2.2 Future Directions

Brush-forming PAA's can be utilized in a wide range of different materials science and biomaterials/biomedical applications. In addition to alumina particles, other metal oxides may be sterically stabilized with brush-forming PAA's, including silica [2, 3,4] and titanium dioxide.[5, 6] Other colloidal systems may be investigated, where anchor block candidates for each surface can be determined through homopolymer adsorption studies or the screening of combinatorial polypeptide libraries.[7]

As mentioned, brush-forming PAA's may also play a role in several biomaterials applications. One possible application is the design of specialty coatings that will modify a surface to increase its biocompatibility. For example, the Arg-Gly-Asp (RGD) motif is well known for its recognition by integrin receptors and has been frequently introduced into polymeric materials to enhance their cell attachment abilities for use in tissue engineering.[8, 9, 10, 11] It is proposed that the cloning strategy developed within this project could be used to design optimized PAA's for specialty coatings resulting in brush layers that display the RGD motif in order to promote cell attachment. A well-defined brush layer would offer a higher level of control over the interactions at the material-cell interface.

For biomedical applications, optimized brush-forming PAA's may play a role in several areas, including drug delivery and biosensors. Due to the development of gene therapy as a promising method of treatment for various types of tumors and genetic diseases, there has been strong interest in the designing of nonviral polymeric-based gene vehicles to deliver therapeutic DNA to target tissues.[12, 13, 14] Therefore, it is proposed that the methods to design brush-forming PAA's presented in this work can also be adapted to design PAA's tailored for gene delivery systems.

Recent advances in biosensors have been through the immobilization of bioactive molecules on surfaces such as modified glass slides (protein chip),[15] single walled carbon nanotubes,[16] semiconductor nanocrystals (or quantum dots),[17] gold nanoparticles[18] and

magnetic nanoparticles (Fe_3O_4)[19]. The results in Chapter 4 demonstrate how brush-forming PAA's can be used to tether proteins to a specific surface. It is, therefore, possible to imagine using the methods described in this work to design brush-forming PAA's that tether bioactive proteins that will have some specific function or activity that can be developed into a novel biosensor or bioanalytical probe.

The fact that the cloning strategy introduced is compatible with a variety of different expression systems also opens the door for future experiments. As mentioned, the cloning strategy can be used to produce sequentially modified recombinant proteins in prokaryotic, yeast and mammalian hosts. Therefore, this method could be used to investigate any number of biochemical processes. For example, this method could be used to sequentially add domains to a protein and study the affect on the protein folding and secretion pathways in *P. pastoris*. Hence, the versatility of the cloning method is not limited only to prokaryotic systems.

7.3 Conclusion

The design and discovery of new surface-active polymers that self-assemble on a solid substrate to form brush layers will have a major impact on numerous bioengineering and materials science applications. Through recombinant DNA technology, there exists the potential to use the natural protein synthesis machinery to design such polymers with an exactly specified amino acid sequence, thus controlling the polymer's composition and structure at a level unequaled by conventional organic polymer synthesis techniques. As stated, the objective of this project was achieved with the development of a new cloning strategy customized for the rapid production of unique poly(amino acids) that are surface-active and self-assemble on metal oxide surfaces to form brush layers.

The presented research is innovative in its synergistic combination of biotechnology, polymer science and surface science to produce and evaluate novel polymers. At the conclusion of this project, we have demonstrated that the newly developed cloning strategies can be easily applied to the production of self-assembling PAA's. The simplicity of the strategy means that research groups will no longer need to develop their own customized molecular biology reagents. Therefore we, in addition to other groups, will be able to rapidly produce these PAA's and contribute to both

fundamental and applied studies in PAA self-assembly phenomena. We believe that successful completion of this work is a significant achievement in the fields of biopolymers and biomaterials.

7.4 References

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