

# 博士論文（要約）

## Development of Novel Elastin-Like Block Polypeptides and Their Use in Construction of Biomimetic Extracellular Matrices

(新規なエラスチン類似ブロックポリペプチドの開発と  
それを利用した生体模倣細胞外マトリクスの構築)

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In this dissertation, development of novel elastin-like polypeptides (ELPs) with a “double hydrophobic” block pattern and their use in construction of biomimetic extracellular matrices (ECMs) are described. ELPs are chemically or genetically synthesized biopolymers taking inspiration from elastin, an ECM protein that confers elasticity and resilience to connective tissues. Inheriting the remarkable elasticity and self-assembly properties from the parent protein, these polypeptides have potential applications in the construction of bioelastic artificial ECMs (aECMs) in tissue engineering. The backgrounds on construction of biomimetic ECMs as well as the uses of ELPs in this field are provided in chapter 1. In chapter 2, the construction of various novel block ELPs inspired from the non-uniform distribution of hydrophobic domains in elastin molecule is described. In chapters 3 and 4, the self-assemblies of various ELPs into nanofibers in aqueous solutions are discussed. Preparation and characterization of biomimetic ECMs developed from the obtained ELP nanofibers are discussed in chapter 5. Finally, conclusions and perspectives of the dissertation are given in chapter 6.

In chapter 1, the background and the aim of this dissertation are described. ECM is an intricate meshwork of biomolecules arranged in unique and tissue-specific architectures to function as a house for cells to live and develop properly. An aECM should mimic similar structures and functions of the native one in order to effectively promote tissue regeneration. In constructing elastic aECMs, ELPs are promising materials due to their remarkable mechanical properties. Typically, the hydrophobic repeating motif (Valine-Proline-Glycine-X-Glycine) (VPGXG, X: any amino acid except proline) found in the elastin molecule (tropoelastin) is used in designing ELPs. In aqueous solutions, poly(VPGXG) assembles into nanoparticles by hydrophobic interaction in response to increase of temperature. However, poly(VPGXG)-containing ELPs were unable to form nanofibers due to the lack of directional interactions. Novel ELPs having fiber formation ability as well as well-defined mechanical properties and biological activities will expand the use of ELPs in mimicking elastin organizations. The aim of this dissertation is to develop novel ELPs with those desired properties for the construction of biomimetic ECMs.

In chapter 2, the construction of ELPs with a novel block structure is described. ELPs mimic the repeating motifs in hydrophobic domains of tropoelastin because of their importance in mechanical properties and self-assembly. There are two types of hydrophobic domains, the proline-rich domains with (VPGXG) repeating motifs, mainly found in the middle region, and the glycine-rich domains with ( $Z_1GGZ_2G$ ) ( $Z_1, Z_2$ : V or L) repeating motifs, mainly found in the two termini of tropoelastin. While poly(VPGXG) assembles into nanoparticles by hydrophobic interaction, poly( $Z_1GGZ_2G$ ) is

prone to form short fibrils by directional hydrogen bonds. The localization of these domains might be important in directing the assembly of tropoelastin into fibers; however, it has not been explored yet. Herein, “double hydrophobic” block ELPs, mimicking the non-uniform distribution in tropoelastin, were constructed for the first time. Those are a triblock **GPG** and a diblock **PG**, in which a proline-rich sequence (VPGXG)<sub>25</sub> (X: V or F) is conjugated by a glycine-rich sequence (VGGVG)<sub>5</sub> at two termini or at the C-terminal, respectively. Functionalized block ELPs were also constructed by inserting cross-linking sequences (KAAK) and a cell-binding sequence (GRGDS). **X-GPG** is the triblock **GPG** with an additional KAAK sequence at the C-terminal for enhancing cross-linking propensities. **F-GPG** is the triblock with cross-linking sequences at the conjunction of the block components as well as two termini, and a cell-binding sequence at the C-terminal for interacting with cells. The DNA plasmids encoding these block ELPs were constructed by cloning techniques and transformed into *E. coli* for expression. The successful synthesis and purification of these ELPs were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF-MS).

In chapter 3, self-assembly of **GPG** and **PG** in aqueous solutions induced by several factors is described. The effects of temperature on the self-assembly of **GPG** in water were examined at first. Changes in secondary structures and morphologies of **GPG** were studied by circular dichroism (CD) spectroscopy and atomic force microscopy (AFM), respectively. At low temperature (16 °C), **GPG** showed no structural changes after 7 days. By contrast, the triblock polypeptide initially assembled into nanoparticles rich in  $\beta$ -turns, which further connected into beaded nanofibers along with an increase in  $\beta$ -sheets at 45 °C after 7 days. Interestingly, the obtained fibers showed curled or coiled appearance, indicating considerable flexibility. Also, they were well-dispersed in water even after prolonged incubation at 45 °C. The diblock **PG** showed similar fiber formation but with lower  $\beta$ -sheet contents. On the other hand, the block components **P** and **G** only assembled into nanoparticles and short fibrils, respectively. This result indicates the importance of the block structure on the fiber formation.

Effects of addition of an organic solvent 2,2,2-trifluoroethanol (TFE) were also studied because it affects the self-assembly of **GPG** differently from the effect of temperature only. TFE disrupts the hydration water molecules and lowers the transition temperature of **GPG**. The solvent also induces and strengthens the hydrogen bonds, which are important for the fiber formation, by providing low dielectric environments. Herein, effects of various TFE concentrations on the assembled structures

of **GPG** were systematically investigated. Addition of 10–30% (v/v) TFE induced beaded fiber formation similar to that in the water system but with faster manner (1 day) at 16 °C, the temperature that **GPG** could not form fibers in water. The  $\beta$ -turn and  $\beta$ -sheet contents in **GPG** fibers were also promoted along with the increase of TFE concentrations according to the CD spectra. On the other hand, **GPG** only formed aggregates of nanoparticles in 60% (v/v) TFE due to the formation of intramolecular hydrogen bonds in  $\alpha$ -helices. These results suggest that the assembled morphologies can be controlled by formation of inter- or intra-molecular hydrogen bonds at various TFE concentrations.

In summary, **GPG** assembles into homogeneous beaded nanofibers with flexible appearance and excellent dispersibility in water at high temperature (45 °C) as well as in TFE aqueous solutions at 16 °C.

In chapter 4, the self-assembly of **X-GPG** and **F-GPG** in aqueous solutions was studied to find the conditions for the fiber formation of these polypeptides. The self-assembly was conducted in water at 45 °C, 7 days at first. **X-GPG** showed beaded nanofiber formation similar to that of **GPG** along with the increase of  $\beta$ -turns and  $\beta$ -sheets according to the AFM and CD spectra results. On the other hand, **F-GPG** only formed aggregates after 7 days and even at the prolonged incubation (10 days). Therefore, TFE was added to induce the formation of **F-GPG** fibers. Only few thin fibers were formed within bigger aggregates at 16 °C, after 1 day. The temperature was raised to 25 °C in anticipation of additional temperature-triggered self-assembly. Still, **F-GPG** fibers were detected together with aggregates in 10–30% TFE. The presence of cross-linking sequences with charged amino groups near the **G** block might strongly reduce the  $\beta$ -sheet forming ability, resulting in the decreased propensity of **F-GPG** to form fibers.

Chapter 5 reports the preparation of biomimetic ECMs using the obtained ELP nanofibers in chapters 3 and 4. To improve the stability of ELP nanofibers, cross-linking of **X-GPG** fibers by BS3, an amine to amine cross-linker, were performed. The resulting cross-linked fibers remained stable at 16 °C, after 2 days while the non-cross-linked counterparts mostly dissociated. The cross-linked fibers, which are composed of biocompatible components and stable over a wide range of temperature, show promising applicability in mimicking elastin organizations.

aECMs from the obtained **GPG** nanofibers and gelatin were also constructed to mimic the fibrous structures in skin dermis, where small amount of elastin fibers effectively control elasticity of the tissue. Composite fibrous films of gelatin and a low content (< 1 wt%) of **GPG** fibers were obtained by electrospinning mixtures of these components in 30% TFE aq. solutions at the optimized conditions.

Scanning electron microscopy showed that both the gelatin and **GPG**/gelatin composite films have fibrous morphologies with diameter of about 100 nm. The films were cross-linked by glutaraldehyde vapor for 16 h at room temperature. They were then washed carefully and subsequently cut into a dumbbell-shape for tensile tests to determine elastic moduli. The cross-linked **GPG**/gelatin composite films showed decrease in the elastic moduli compared to that of gelatin alone, suggesting that the mechanical properties of the composite materials could be controlled by **GPG** fibers.

In conclusion, the construction of various novel block ELPs mimicking the non-uniform distribution of two different types of the hydrophobic domains found in tropoelastin is reported (Chapter 2). The “double hydrophobic” triblock **GPG** and diblock **PG** show the ability to form flexible beaded nanofibers with excellent dispersibility in a wide range of experimental conditions (Chapter 3). **X-GPG**, which contains an additional KAAK sequence at the C-terminal, similarly forms beaded nanofibers in water at 45 °C while **F-GPG**, which contains many additional KAAK within the molecule and a cell-binding sequence at the C-terminal, has a relatively low fiber forming ability due to its high contents of charged cross-linking sequences (Chapter 4). **X-GPG** fibers can be stabilized by cross-linking by the biocompatible cross-linker, BS3, to prevent the dissociation of fibers at low temperature, improving the applicability of ELP nanofibers (Chapter 5). Besides, **GPG** fibers were combined with gelatin to construct the aECMs mimicking the composite fibrous structure in skin dermis by electrospinning. The composite materials show a decrease in elastic moduli by adding a small amount of **GPG** fibers, indicating the function of **GPG** fibers in tuning the mechanical properties of the composite materials (Chapter 5). The results in this research may provide not only innovative ideas for designing peptide-based materials but also opportunities for developing novel elastin-based materials useful for tissue engineering.