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Designing protein-based biomaterials for medical applications ☆

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ABSTRACT

Biomaterials produced by nature have been honed through billions of years, evolving exquisitely precise structure–function relationships that scientists strive to emulate. Advances in genetic engineering have facilitated extensive investigations to determine how changes in even a single peptide within a protein sequence can produce biomaterials with unique thermal, mechanical and biological properties. Elastin, a naturally occurring protein polymer, serves as a model protein to determine the relationship between specific structural elements and desirable material characteristics. The modular, repetitive nature of the protein facilitates the formation of well-defined secondary structures with the ability to self-assemble into complex three-dimensional architectures on a variety of length scales. Furthermore, many opportunities exist to incorporate other protein-based motifs and inorganic materials into recombinant protein-based materials, extending the range and usefulness of these materials in potential biomedical applications. Elastin-like polypeptides (ELPs) can be assembled into 3-D architectures with precise control over payload encapsulation, mechanical and thermal properties, as well as unique functionalization opportunities through both genetic and enzymatic means. An overview of current protein-based materials, their properties and uses in biomedicine will be provided, with a focus on the advantages of ELPs. Applications of these biomaterials as imaging and therapeutic delivery agents will be discussed. Finally, broader implications and future directions of these materials as diagnostic and therapeutic systems will be explored.

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1. Introduction

The evolution of biological systems has created a rich landscape of protein-based materials. Proteins possess a variety of genetically encoded structures and properties spanning many length scales, and an elegantly evolved biological system of production. Extensive studies over the last two decades have elucidated precise structure–function relationships for many proteins; an excellent example of this is elastin, a protein that imparts elasticity to a variety of tissues. Identifying the primary amino acid sequence and subsequent secondary structure of elastin has allowed an exquisite level of control over the thermal and mechanical properties of this material [1]. Utilizing nature's machinery, recombinant polypeptides based on elastin and other proteins can be synthesized with a higher degree of specificity and control than is achievable by chemical methods. As a result, recombinant elastin-like polypeptides (ELPs) can be engineered to possess properties that are highly amenable to applications in biomedical diagnostic imaging and targeted therapeutic delivery [2]. Synthetic polymers, such as poly(caprolactone) (PCL), poly(D,L-lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) have been extensively employed [3], but lack the chemical flexibility, biodegradability, and thermal targeting and release mechanisms that can be exhibited by elastin-based materials. Triggered release mechanisms exhibited by poly(oxylalkylene) block copolymers [4] are similar to the lower critical solution temperature (LCST) phenomena seen in elastin-like materials, but lack the chemical diversity possible with elastin. The precise control over structure, general biocompatibility and ease of functionalization make natural protein-based biomaterials, and elastin-based materials in particular, excellent candidates for biomedical applications.

This review will highlight recent work in the synthesis and purification of protein-based materials, with a focus on elastin-based materials. Methods for designing assembly of proteins into 3-D architectures that would be useful for targeted imaging and therapeutic delivery are discussed. Special note is made of recent work to incorporate non-canonical amino acids and other biological motifs, as well as inorganic components, into elastin-like peptides and other biomaterials, extending the range and applicability of these unique biopolymers. Applications in bioimaging and therapeutic delivery are explored, with an emphasis on the use of ELPs in interrogating and treating pathologies such as cancer and

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cardiovascular disease. Finally, recent advances in imaging systems and targeting ligands will be briefly reviewed, and the potential for protein-based materials to be exploited in these applications will be discussed.

2. Elastin-like polypeptides and other recombinant proteins

Protein biopolymers have typically been modeled after structural proteins such as silk, collagen and elastin. Elastin is a well-known extracellular matrix protein that provides elasticity to a variety of tissues such as blood vessels, ligaments, lungs and skin. Originally derived from tropoelastin [5], elastin is composed of conserved pentapeptide repeat units with the classic form poly(Val-Pro-Gly-Val-Gly) [6]. The conserved peptide sequence found in mammalian elastins has been extensively studied to determine what essential components are useful for specific biomedical applications [7,8]. These polypeptides can interact to form fiber networks and other 3-D structures with controlled properties [9–13]. Synthesis of amphiphilic block polypeptide chains enables assembly mechanisms such as coacervation, a LCST phenomenon. At an elevated transition temperature (T_t), interactions between hydrophobic domains enable self-assembly of higher-order structures, primarily determined by the peptide sequence [14,15]. The loss of entropy due to the formation of the ordered structure is counterbalanced by release of ordered water from the peptide backbone, particularly aliphatic residues [16,17]. The self-assembly process can be affected by other environmental factors, such as protein concentration [18], salt concentration and pH [19–21]; these can be controlled, along with peptide sequence, to produce novel secondary structures and 3-D architectures for a variety of biomedical applications.

2.1. Synthesis of recombinant polypeptides

The genetically encoded synthesis of polypeptide sequences provides a unique level of control over the molecular weight, sequence and stereochemistry of the polypeptide [22], properties that can be difficult to control in chemical polymers [23]. These factors, combined with tunable mechanical properties, natural biocompatibility and the established biodegradation profile [24] of peptide-based materials greatly enhances their potential. Many techniques exist for producing the desired amino acid sequence and molecular weight polypeptides for specific applications.

Initial methods for the production of ELPs focused on the simultaneous generation of a library of oligomeric genes by a concatemerization of a monomer gene [25–27]. Although this process is rapid, precise control over the oligomerization process is not maintained, and the yield contains an oligomer population with a statistical distribution of different lengths. While this may be acceptable in screening studies, more precise methods of generating repeat units for polypeptides of a certain chain length have been investigated.

A more controlled method of producing repetitive gene sequences with a specific molecular weight was developed by Meyer et al. [22] and termed "recursive directional ligation" (RDL). This method utilizes stepwise oligomerization with monomer DNA containing distinct recognition sequences at each end, cut by respective restriction endonucleases. This process produces complementary overhangs with no interruption of the repeat sequences; the two complementary ends are cohesive and ligated into a linearized vector cut by one of two restriction endonucleases, resulting in two repeats of monomer DNA in the vector. This procedure is performed recursively to grow the number of repeats of monomer DNA until the desired number of repetitive genes is achieved. However, this method is limited to specific biopolymer sequences, as the endonuclease restriction site overlaps the coding region. Furthermore, significant background can develop from clones lacking an insert due to self-ligation or incomplete digestion of a vector, reducing cloning efficiency. This method was optimized by McDaniel et al. [28] through recursive directional ligation by plasmid reconstruction (PRe-RDL), in which two halves of a parent plasmid are ligated together, resulting in a dimerized oligomer and reconstitution of a functional plasmid (Fig. 1). This method uses type II restriction endonucleases, which are applicable to any arbitrary oligonucleotide sequence, and produces a seamless junction between repeat peptides. A functional plasmid is only produced in the case of successful ligation, which decreases background from self-ligation and increases efficiency by preventing circularization of the insert.

Another recently developed method, termed overlap extension rolling circle amplification (OERCA) overcomes some of the limitations of the above techniques. Developed by Amiram et al. [29], this rapid, robust and high-throughput method utilizes circular single-stranded DNA and polymerase chain reaction methods to amplify repetitive sequences from a circular gene template. OERCA produces high-yield and high-fidelity repetitive gene libraries, ranging from 0.8 to 1.5 kb, with tunable distributions dependent upon the size range of the OERCA products before ligation. Synthesis of extensive gene libraries has enabled investigation of previously inaccessible non-canonical ELP polymers. However, the PRe-RDL method is often used to produce products with precise control over the final molecular weight of the ELP.

The completed expression vector is commonly transformed in *Escherichia coli*, which is typically used for expressing recombinant proteins with tandem repeats due to its deficiency in homologous recombination [30]. For most applications, it is important to produce recombinant ELPs in large quantities and high purity in a cost-effective manner. As ELPs and ELP-fusion proteins contain disproportionate amounts of glycine, valine, proline and alanine compared to most cellular proteins, it was found that the synthesis could by optimized through the addition of supplemental amino acids. A higher concentration of proline and alanine during the expression process increased the final protein yield up to 3-fold over controls. This can result in yields of up to $\sim$400 mg l^{-1}, a considerable improvement over unoptimized systems [31]. However, expression with *E. coli* systems still suffers from a variety of limitations, including the lack of eukaryotic post-translational systems, insolubility of the overexpressed mammalian proteins and subsequent sequestration into inclusion bodies, difficult purification from cellular contaminants, and endotoxin contamination. Endotoxin has been a specific concern for ELP expression, as it becomes associated with the protein product on cell lysis and is difficult to remove. Recently, yeast and plant [32] expression systems have been explored, with yeast offering the attractive advantage of ease of incorporation into industrial-scale fermentation systems. However, protein yields are often low when compared to *E. coli*, and Sallach et al. [33] have investigated a novel strategy to construct a gene with enhanced sequence diversity that encodes a highly repetitive elastin-like protein polymer for expression in *Pischia pastoris*. A modified concatemerization strategy was designed in which seven dissimilar monomer repeat units, encoding identical pentapeptide repeat sequences, served as a monomer library for the concatemerization reaction. This strategy can be used to create large, repetitive genes for a variety of expression systems with the potential to generate glycosylated ELPs.

Purification of ELPs has been investigated through a number of different methods, and affinity chromatography typically allows one-step purification. Although useful in laboratory-scale operations, this can represent a significant cost in scaled-up production of the final protein. Because ELPs undergo a reversible inverse temperature transition, it is possible to exploit this property to purify

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