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Stimulus responsive elastin biopolymers: applications in medicine and biotechnology

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Abstract

Elastin-like polypeptides (ELPs) are artificial polypeptides, derived from Val-Pro-Gly-Xaa-Gly (VPGXG) pentapeptide repeats found in human tropoelastin, that reversibly coacervate above a critical temperature. Genetically encodable ELPs are monodisperse, stimuli responsive, and biocompatible, properties that make them attractive for drug delivery and tissue engineering. The potential of ELPs to self-assemble into nanostructures in response to environmental triggers is another interesting feature of these polypeptides that promises to lead to a host of new applications.

Introduction

ELPs are artificial biopolymers composed of the pentapeptide repeat Val-Pro-Gly-Xaa-Gly (VPGXG), which is derived from the hydrophobic domain of tropoelastin. At low temperatures, ELPs are soluble in aqueous solution, but as the solution temperature is raised, they become insoluble and aggregate at a critical temperature, termed the inverse transition temperature (T_i) [1–3], a phenomenon similar to the lower critical solution temperature transition [4,5]. This process is typically reversible so that subsequent cooling of the solution below the T_i results in the resolubilization of the ELP. The inverse phase transition can also be isothermally triggered by the addition of salt, and the response of ELPs to different salts follows the Hofmeister series, which ranks salts according to their ability to precipitate proteins out of aqueous solution [1,6–8]. ELPs can also be designed to respond to other physical stimuli such as redox, pH, light, etc. [9–11] by incorporation of suitable guest residues in the polypeptide chain at the fourth position.

ELPs are useful for a wide variety of biomedical applications for several reasons. First, the T_i of ELPs can be precisely tuned between 0–100 °C with a precision of a few degrees Celsius. This allows ELPs to be rationally designed that are optimized for a specific application (e.g. drug delivery, protein purification, etc.) [12]. Second, ELPs can be synthesized as monodisperse polymers in *Escherichia coli* from synthetic genes. The precisely defined molecular weight (MW) of ELPs is ideal for drug delivery because MW is a key parameter that controls both the route of clearance from the body and the *in vivo* half-life of a polymer [13]. Third, ELPs can be expressed at gram per liter quantities in a laboratory setting, so that they can be conveniently produced at a cost that rivals that of synthetic polymers [14]. Fourth, the thermal responsiveness of ELPs permits their purification without complex chromatographic separation, simply by exploiting the phase

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transition behavior of ELPs [15]. Fifth, ELPs appear to be biocompatible [16], suggesting their suitability for *in vivo* applications.

The study of ELPs was pioneered by Dan Urry, who synthesized a large number of polypeptides over the course of three decades and studied their biophysical properties in solution and as cross-linked elastomeric materials. These studies have been summarized in many reviews [1,17–19]. This era of ELP research was distinguished by two features: polypeptides that were chemically synthesized; and applications of these polypeptides largely as bulk, cross-linked materials. With the advent of molecular biology, a new era in the synthesis and application of ELPs and other repetitive polypeptide materials has dawned, which is characterized by recombinant synthesis of precisely defined polymer architecture. In the remainder of this review, we briefly summarize recent applications of recombinant ELPs, with an emphasis on applications at the molecular to meso-length scale.

Purification of ELP-fusion proteins

In 1999, our group published the first paper that demonstrated that the inverse transition behavior of ELPs was imparted to ELP fusion proteins [15], and we exploited this finding to develop a protein purification process that we have named inverse transition cycling (ITC) [12,14,15,20•,22,23••]. This approach for protein purification has subsequently been validated by other groups [24–31,32••]. In ITC, the phase transition of the ELP fusion protein is triggered either by adding NaCl or by raising the temperature, and the aggregated ELP fusion proteins are separated from other contaminants in the cell lysate by centrifugation. The supernatant containing *E. coli* biomolecules is discarded and the pellet of aggregated ELP is redissolved in cold buffer. This step is typically followed by centrifugation below the T_t of the ELP fusion protein to remove insoluble contaminants that may have been trapped in the pellet of the ELP fusion protein during its phase transition triggered aggregation. The steps described above can be repeated until the fusion protein is purified to the desired level. As an alternative to centrifugation, aggregated fusion proteins can be separated from contaminants by filtration [33] where the micron-sized aggregate is retained on the membrane and eluted by cold low-salt buffer.

ITC has many desirable attributes: it is inexpensive, as it requires no special equipment or resins and only uses inexpensive reagents such as sodium chloride to trigger the inverse phase transition. Multiple rounds of ITC increase the purity of the ELP fusion protein, and target proteins can be liberated from their ELP purification tag either by proteolytic cleavage at engineered polypeptide sequences [15,21,22,23••] or by self-cleaving inteins [32•,33]. ITC can also be easily multiplexed and scaled up, as it is a batch process.

The nature of the protein in the fusion construct has an effect on the inverse transition temperature of the ELP fusion protein. Hence *a priori* prediction of the T_t is desirable to optimize the purification of ELP-fusion proteins by ITC. We have found that the change in T_t of the fusion compared with the free ELP [fusion $\Delta T_t = T_t$ (ELP fusion protein) – T_t (free ELP)] depends on the balance of solvent-accessible hydrophobic surface area of the protein to charged surface area [23••]. Proteins with a large fraction of solvent-accessible hydrophobic surface area depress the T_t of the fusion protein relative to the free ELP. In contrast, proteins that have a large number of solvent accessible ionizable residues raise the transition temperature relative to free ELP. Typically, these two effects balance each other in many proteins, so that the ΔT_t effect is small. However, for several proteins we have observed that the ΔT_t parameter can be as much as $\sim \pm 15$ °C, when one of these two factors dominates the surface of the protein. An interesting example is the fusion of ELP and tendamistat, a small (~7 kDa) protein inhibitor of porcine pancreatic α -amylase (PPA). Despite its small size, tendamistat has a large fraction of exposed hydrophobic surface area

(43%) but few ionizable residues, so that its ELP fusion exhibits a large fusion ΔT_t effect of -17°C . The hypothesis that this depression in the T_t relative to the free ELP is caused by its large fraction of hydrophobic surface area was validated by the observation that binding of PPA to tendamistat buries much of the exposed hydrophobic surface area in tendamistat and increases the transition temperature by 7°C . We are now working on developing a detailed and quantitative understanding of the fusion ΔT_t effect as it will allow *a priori* optimization of ITC for specific proteins, and also because this effect can itself be exploited to drive the phase transition by protein–ligand interactions, which would provide a new and exciting class of biochemically relevant triggers to drive the phase transition of ELP fusion proteins.

Affinity capture by ELP conjugates

The fact that the non-covalent complex of tendamistat-ELP and PPA also exhibits a phase transition is interesting because it demonstrated that non-covalently bound complexes also undergo the phase transition. Chen and colleagues have independently exploited this feature to demonstrate that a protein A–ELP fusion can be used to capture antibodies from solution, which provides an alternative to protein A chromatography for the purification of antibodies [24,25].

Block co-polymers

ELPs are particularly attractive molecules to synthesize block copolymers that self-assemble into polymer micelles, because of the exquisite control over the polypeptide sequence afforded by recombinant synthesis that is the primary determinant of self-assembly. In the original work in this area, Lee *et al.* synthesized an elastin-mimetic di-block copolymer using VPGEG-(IPGAG)₄ and VPGFG-(IPGVG)₄ as the hydrophilic block and hydrophobic block, respectively [34]. Temperature-dependent formation of micelles was verified by dynamic light scattering (DLS), and differential scanning calorimetry (DSC) was used to measure the enthalpy of self-assembly. Subsequently, a tri-block copolymer was prepared using the same hydrophilic block capped on both ends by hydrophobic blocks using VPAVG-(IPAVG)₄, and conventional TEM images showed the formation of spherical and cylindrical worm-like micelles [35]. Interestingly ^1H - ^{13}C heteronuclear correlation multiple quantum coherence NMR spectroscopy demonstrated that hydrophobic block residues (isoleucine and alanine) become motionally restricted above the T_t , while the hydrophilic block residues do not, suggesting temperature-dependent formation of hydrophobic nanodomains [35].

We have also synthesized similar di-block copolymers without any ionizable residues and shown that these ELP block copolymers exist in at least three or more distinct phases: unimer at temperatures below the T_t of the hydrophobic block, micelle at temperatures intermediate between the T_t of both blocks and micron sized aggregates at temperatures greater than the T_t of the more hydrophilic block. More recently, we have found that for a di-block ELP to form a spherical micelle, there must be a significant difference between the T_t of the hydrophobic and hydrophilic blocks, as well as a MW ratio of 1:2 to 2:1 between the two blocks (MR Dreher, PhD thesis, Duke University, 2006). These di-block ELPs show considerable promise for drug delivery, although much work remains to be done in understanding their physicochemical behavior before they can fulfill this promise.

Drug delivery

The ability to synthesize ELPs with a precise MW and low polydispersity, the potential biocompatibility of ELPs, and their controlled degradation make them interesting delivery vehicles for systemic drug delivery. The use of ELPs as soluble drug carriers for the treatment of solid tumors has been the focus of much of our work. The tunable T_t of ELPs

has been exploited for noninvasive thermal targeting to solid tumors when combined with hyperthermia treatment — the application of mild heat to the site of the tumor to promote uptake of ELP–drug conjugates within tumors.

Our thermal targeting strategy is to design an ELP that has a T_t that is between body temperature and 42 °C, a temperature that is approved for clinical hyperthermia. We have shown in experiments in nude mice that were implanted with subcutaneous human tumors, that a thermally responsive ELP accumulates in tumors heated to 42 °C to a twofold greater extent as compared with tumors that were not heated [36]. It is important to note that this gain in tumor accumulation is in addition to the enhanced accumulation that ELPs, like all macromolecular carriers exhibit in tumors compared with small molecule therapeutics because of the enhanced permeability and retention effect [37]. Furthermore, *in vitro* cell culture studies show a similar twofold enhancement in the uptake of the ELP by heated tumor cells compared with cells maintained at 37 °C [38]. We are currently exploiting the thermally triggered phase transition of ELP to deliver a conventional chemotherapy drug, doxorubicin, to tumors. Doxorubicin was functionalized by a hydrazone linker, and attached to ELP via a carboxy-terminal cysteine residue [39,40]. Following systemic administration and cell uptake into low pH compartments such as endosomes and lysosomes, the pH sensitive hydrazone linkage is cleaved and regenerates free drug.

Additional strategies have been recently described for delivery of drugs using ELP carriers. Herrero-Varrell *et al.* have synthesized micron-sized aggregates of VPAVG repeats that exhibited pronounced hysteresis of 12.8 °C between heating and cooling [41]. Dexamethasone phosphate (DMP), a model drug, was physically entrapped within the ELP microparticles. Although the presence of the drug affected the T_t , aggregate formation was not affected from a size or morphological standpoint. Unlike previously engineered ELP nanoparticles, the formation of these micron-sized aggregates is not reversible as they will stay intact at temperatures below the T_t , reducing the need for precise temperature control during injection and administration.

The biodegradation and biocompatibility of ELPs are suitable for preparing bulk implants/injectables. These implants can hold cells, providing a substrate for tissue engineering. Alternatively, these formulations are suitable for encapsulation and release of proteins, DNA [42] or drugs [41]. In one approach, Betre *et al.* evaluated ELPs that aggregate below body temperature as a potential injectable depot for intra-articular drug delivery [43•]. Biodistribution studies revealed that the aggregating ELP has a 25-fold longer half-life in the injected joint than an equivalent molecular weight ELP that remains soluble and does not aggregate. These results suggest that the intra-articular delivery of ELP fusion proteins may be a viable strategy for the prolonged release of protein drugs for osteoarthritis.

In an alternative approach, injectable depots of silk-ELP hybrids were formed *in situ* for local delivery of DNA. Megeed and co-workers [42] designed a biopolymer with alternating blocks of silk-like (GAGAGS)_n and ELPs. Upon heating, the silk-like blocks adhere to each other to form a stable hydrogel with a swelling ratio ~10. Plasmid DNA was encapsulated within these hydrogels, and the constructs released active DNA over a period of at least two weeks. When directly injected into a tumor grown in a mouse model, the reporter gene product luciferase was detectable for three weeks after implantation [42]. This approach demonstrates the potential for sustained release of DNA from ELP depots, and may also be applicable for the release of other high molecular weight species such as proteins.

Tissue engineering

In collaboration with the group of Lori Setton at Duke University, we have investigated the use of ELPs for cartilage tissue engineering. ELP coacervates promote the synthesis and

retention of cartilaginous matrix from encapsulated cartilage cells and adult stem cells when cultured *in vitro* [43•,44•]. These results demonstrate that ELPs can provide the appropriate physical and biochemical environment to maintain chondrocyte differentiation and support cartilage matrix synthesis *in vitro* and suggest the potential utility of ELP to serve as a scaffold for cartilage repair.

ELPs have been chemically cross-linked to form hydrogels suitable for tissue engineering. The maximum shear modulus of these materials (310 kPa) [16,45,46] compares favorably with that of normal cartilage of (~440 kPa). We also investigated a bioinspired, *in situ* cross-linking method to form ELP hydrogels by covalent cross-linking between lysine and glutamine guest residues catalyzed by tissue transglutaminase [44•]. A significant increase in the accumulation of sulfated glycosaminoglycans was observed following encapsulation of chondrocytes in these enzymatically cross-linked hydrogels. Histological sections revealed the accumulation of a cartilaginous matrix rich in type II collagen and lacking in type I collagen, indicative of hyaline cartilage formation. These results provide evidence of chondrocytic phenotype maintenance for cells in the ELP hydrogels *in vitro*. Additionally, the dynamic shear modulus of ELP hydrogels seeded with chondrocytes increased from 0.28 to 1.7 kPa during a 4-week culture period, which suggested restructuring of the ELP matrix by deposition of functional cartilage extracellular matrix components.

In an alternative approach, several studies have demonstrated that ELP block copolymers can form physically cross-linked networks simply by triggering the phase transition of one block. This is an attractive strategy for the *in situ* formation of tissue engineering scaffolds, as it eliminates the need for exogenous chemical cross-linkers. As described previously, silk-like blocks alternating with hydrophilic ELPs can non-covalently form hydrogels [47]. Similarly, block copolymers that are solely comprised of ELP blocks can form hydrogels via T_i -triggered physical cross-linking. In a recent example, ELP tri-block copolymers with a hydrophobic-hydrophilic-hydrophobic architecture formed a physically cross-linked network upon T_i -triggered coacervation of the outer hydrophobic blocks [48,49]. Nagapudi and co-workers [48] synthesized a series of ELP tri-blocks with reduced inner block hydrophilicity. Upon T_i -triggered physical cross-linking they found that block copolymers with the most hydrophilic inner blocks formed hydrogels with complex shear moduli ranging from 4.5 to 10.5 kPa. Decreasing the hydrophilicity of the inner-block resulted in the formation of hydrogels with a significantly greater shear moduli of up to 280 kPa [48]. These systematic studies demonstrate that ELP tri-blocks are capable of spontaneously forming hydrogels that have mechanical properties tunable at the genetic level. Such structure–property studies are important because they will provide the design rules for the synthesis of ELP-based scaffolds that have mechanical properties that match a target tissue.

ELPs have also been engineered that promote the attachment of anchorage dependent cell lines [50•,51–54]. This is crucial for tissue engineering because some cell types, such as human umbilical vein endothelial cells, do not attach to unmodified ELPs. To promote ELP-cell interactions, the fibronectin CS5 cell-binding sequence (EEIQIGHI-PREDVDYHLYPG) was incorporated into an ELP [50•]. Incorporation of CS5 improved cell attachment but was insufficient to promote cell spreading. In contrast, incorporation of a fibronectin-derived RGD peptide (YAVTGRGDSPASSKPIA) into an ELP was sufficient both for cell attachment and spreading [50•]. As demonstrated by these studies, ELPs offer the opportunity to genetically encode at the sequence level materials that form biocompatible hydrogels without chemical cross-linkers, with tunable mechanical properties, and which enable the presentation of biochemical cues within the scaffold.

Conclusions

In this review, we have summarized recent examples that demonstrate the utility of ELPs for a wide range of biomedical applications that exploit their monodispersity, stimuli-responsiveness and biocompatibility. The monodispersity and precisely defined properties of ELPs make them attractive for drug delivery, while the predictable placement of cross-linking groups and binding moieties at specific sites along the polypeptide chain and their programmable degradation rates make them useful for tissue engineering. Finally the potential of ELPs to self-assemble into nanostructures in response to environmental cues, a nascent area of research, will lead to a host of new applications of these recombinant polymers.

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