Biological Applications of Elastin Like Polypeptides and the Inverse Transition Cycle in the Pharmaceutical Industry- A Review

A Thesis Submitted By

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Student ID: MS-170728

Session:2016-17

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Biotechnology and Genetic Engineering Discipline

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Bangladesh

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March, 2022

DEDICATED

TO MY.....

BELOVED MOTHER & FATHER

Biological Applications of Elastin Like Polypeptides and the Inverse Transition Cycle in the Pharmaceutical Industry- A Review

Declaration

This thesis paper is the original thesis work of the author, except as otherwise stated. The above thesis work or any part of this work has not been submitted in anywhere for the award of any degree or diploma or any other university. The findings of the research have not been/ will not be published anywhere without the concurrence of the concerned supervisor.

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ABSTRACT

Proteins are essential throughout of the biological and biomedical sciences and the purification strategies of proteins of interest have advanced over centuries. Elastin Like Polypeptides (ELPs) are large molecular and stimulus-responsive biopolymers derived from human elastin. Recent research has identified the various applications of ELPs as nanomedicines and Inverse Transition Cycle (ITC) are far from being completely understood. This review provides a comprehensive idea about applications of ELPs and ITC. ITC is considered an inexpensive and efficient way of purifying recombinant ELP fusion proteins. ELPs have been used extensively for protein purification, tissue engineering, drug delivery in a variety of different embodiments as soluble macromolecular carriers, self-assembled nanoparticles, or thermally coacervated depots. This review focuses on recent advances in understanding valuable the biological application of ELPs and briefly highlights on the possible use of technologies to improve the efficiency of the ITC in the pharmaceutical industry to obtain benefit.

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ABBREVIATION	ELABORATION
ELPs	Elastin like polypeptides
ECM	Extracellular matrix
LCST	Lower critical solution temperature
ITT	Inverse temperature transition
iTEP	immune-tolerant elastin-like polypeptides
Tt	Transition temperature
IMAC	immobilized metal ion affinity chromatography
FAME	Fatty acid–modified ELP
POP	Partially ordered polymer;
SELP	Silk ELP;
ZIPP	Zwitterionic polypeptide.
GLP-1	glucagon-like peptide 1
CVD	cardiovascular disease
EPR	enhanced permeability and retention effect
VIP	vasoactive intestinal peptide
e.g	exempli gratia= for example
etc.	et cet. Era= and the others
°C	Degree celcious

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Elastin is a polymeric extracellular matrix (ECM) protein, found in tissues as varied as the skin, lungs, blood vessels, and cartilage, which provides structural integrity to the tissues and organs of the body. (Badlock et al., 2011). Mainly, Elastin is to provide elasticity for large blood vessels (aorta), ligaments, lungs and skin. Tropoelastin, the soluble precursor to elastin, is composed of alternating hydrophobic and hydrophilic crosslinking domains (Sarah et al., 2009).

Tropoelastin is the fundamental building component of all elastins. Nearly 90% of the elastic fibre consists of elastin protein, which is a polymer of the monomeric precursor, tropoelastin and is the dominant contributor to fibre elasticity (Muiznieks et al., 2010). Further, tropoelastin has the ability to self-assemble under physiological conditions and exhibit an inverse temperature transition (ITT) behavior.

Elastin is produced and developed in early childhood stage, and little or no elastin made by adult tissues. Recently, the use of biopolymer-based elastin and elastin-derived molecules have been increasingly investigated for their application in biomedical and drug delivery systems due to their remarkable properties such as elasticity, self-assembly, long-term stability and biological activity.

The role of elastin in native ECM has made it of particular interest in biological applications such as drug delivery and tissue engineering (Bellingham et al., 2003). Elastin can be applied for biomedical applications in various forms, including insoluble elastin fibres, hydrolysed soluble elastin, recombinant tropoelastin, repeats of synthetic peptide sequences, namely elastin-like polypeptides (ELPs) and as block copolymers of elastin (Daamen et al., 2007).

Elastin-like polypeptides (ELPs) are stimuli-responsive, self-assembling biopolymers consisting of repetitive amino acid sequences inspired from natural elastin. ELPs composed of VPGXG pentapeptide repeats exhibit an inverse temperature phase transition. They are soluble at temperatures below a characteristic cloud point temperature (Tt) (also known as the inverse transition temperature) and aggregate into micron scale coacervates above the Tt. This phase transition occurs over a short time scale and is typically reversible, such that

ELP coacervates will resolubilize when returned to a temperature below the Tt. The Tt is precisely controlled by intrinsic parameters including the composition and molecular weight (MW) of the ELP and is also influenced by extrinsic factors such as concentration, solutes, and, for some ELP sequences, by solution pH (Urry et al., 2004).

ELPs are useful for a wide variety of biomedical applications for several reasons. The LCST of ELPs can be precisely tuned between 0–100°C with a precision of a few degrees Celsius. This allows for the design of ELPs that are optimized for a specific application (e.g., drug delivery, protein purification, etc.). ELPs can be synthesized as monodisperse polymers in E. coli from synthetic genes. The precisely-defined MW of ELP is ideal for drug delivery because MW is the key parameter that controls both the route of clearance from the body and the in vivo half-life of a polymer. ELPs can be expressed at gram per liter quantities in a laboratory setting, allowing ELP production at a cost that rivals that of synthetic polymers. The thermal responsiveness of ELPs permits their purification without complex chromatographic separation simply by exploiting the phase transition behavior of ELPs.

The objectives of this review are:

1. To discuss current biomedical and biotechnological applications of ELPs.
2. To discuss the inverse transition cycle of ELPs.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Definition of Elastin Like Polypeptides

Elastin-like polypeptides (ELPs) are artificial repetitive polypeptides derived from mammalian elastin. They consist of a pentapeptide repeat, Val-Pro-Gly-Xaa-Gly, where Xaa, termed the guest residue, is any natural amino acid except proline (Urry et al., 1992). ELPs are soluble in aqueous solution below their inverse transition temperature (Tt). ELPs are thermally responsive and demonstrate lower critical solution temperature (LCST) phase behavior. ELPs are members of a broader class of stimulus responsive ''smart'' polymers that exhibit inverse temperature phase transition behavior in response to changes in their solution environment. ELPs are not simply temperature-responsive polymers, as their reversible aqueous de-mixing behavior can be isothermally triggered by cosolutes, especially kosmotropic salts, and variants of these polymers can be designed to exhibit this behavior in response to changes in pH, redox triggers, and light (Meyer et al., 2001). ELPs may be more environmentally sensitive, as seen by a direct comparison of the change in their Tt, compared with salt concentration (Urry et al., 1991).

2.2 Tropoelastin

Tropoelastin is a protein, of size 72kDa, that comes together via cross-links to form elastin in the extracellular matrix of the cell. The cross-link formation process is mediated by lysyl oxidase. One of the major reasons that elastin can withstand high levels of stress in the body without experiencing any physical deformation is that the underlying tropoelastin contains domains that are highly hydrophobic. These hydrophobic domains, consisting overwhelmingly of alanine, proline, glycine, and valine, tend towards instability and disorderliness, ensuring that the elastin does not lock into any specific confirmation. Tropoelastin is encoded by a single gene in humans and predominantly laid down in utero and early childhood, providing a durable resource that is intended to elastically serve until old age. This exquisite assembly helps to generate elastic tissues as diverse as artery, lung, and skin. Consequences of elastolytic damage in aortic aneurysms, emphysema, and solar elastosis confirm the key roles of elastin in structure and cellular interactions (Jensen et al.,

2000). These tissues rely on this paradoxical combination of organized tissue structures built from an intrinsically unstructured protein. Tropoelastin serves as a component of rigidly organized assemblies, yet enables the formation of dynamically distensible, elastic tissues.

Tropoelastin is the fundamental building component of all elastins. Tropoelastin, the soluble precursor to elastin, the main elastic protein found in mammals. It is composed of alternating hydrophobic and hydrophilic crosslinking domains (Sarah et al., 2009).

2.3 Properties of ELPs

ELPs originate from human tropoelastin, they are non-injurious, non-immunogenic and are compatible with living systems without causing harm. ELPs composed of VPGXG pentapeptide repeats exhibit an inverse temperature phase transition. The repetitive ELP phenomenon was inspired from W4 domain of human tropoelastin that consists of repetitive VPGG, VPGVG and APGVGV peptides (Rapaka and Urry., 1978). ELPs are appropriate for in vivo applications because of its biocompatibility, biodegradability and non-immunogenic properties (Mackay et al., 2008). Another important property is the ability of ELP to retain its phase transition property when fused to other proteins, the fusion protein can be purified using the thermal nature of the ELPs.

ELPs exhibit a quick and reversible phase transition behavior at a specific temperature referred to as the lower critical solution temperature (LCST), commonly known as the inverse transition temperature (Tt) (Meyer and Christensen., 1999). When the LCST is reached, an ELP assumes a close-to-random coil conformation and becomes highly soluble in aqueous solution. When solution is then heated and LCST is reached, ELPs become insoluble and form large micron-size aggregates that are visible and detectible by the naked eye. This transition is entirely reversible, such that the aggregated polypeptide totally disappears when the temperature is lowered below the LCST of the ELP. The LCST decreases with increasing ionic strength, polymer concentration, and polymer length. The nature of the guest residue also affects the transition temperature, as more hydrophobic amino acid residues lower the transition temperature and vice versa.

2.4 Review of Previous Research Findings

ELPs are useful for a wide variety of biological applications for several reasons. Current biomedical and biotechnological applications of ELPs in the areas of drug delivery, tissue engineering, protein purification, and surface modification.

Wu et al., (1993) described a novel application of electro spraying to construct ELP nanoparticles for drug delivery purposes. Electro spraying, an evolving way for the rapid and high throughput production of nano scale particles of same morphology for controlled drug delivery applications. The encapsulation and activated release of a model drug, Doxorubicin, from ELP nanoparticles. Their results revealed that electro spraying is a productive and flexible method for making stimuli-responsive drug particles that have potential drug delivery applications.

Meyer & Chilkoti et al., (1999) reported that Purification of elastin-like polypeptides (ELPs) and ELP fusions by inverse transition cycling. After cell lysis, ELP coacervation is triggered with the addition of heat and salt. Soluble contaminants are separated from the ELPs by a centrifugation step at a temperature above the transition temperature ($T > Tt$). After the ELP is redissolved in cold buffer, a centrifugation step at $T < Tt$ is performed to remove insoluble contaminants from the ELPs, which are now in solution.

Sarah R. MacEwan et al., (2009) discussed about the genetically encoded design and recombinant synthesis of ELPs that enable precise control of their physicochemical properties and which have led to a wide range of biomedical applications of these biopolymers in the last decade. A protein A-ELP fusion was used to purify IgG and IgM antibodies by indirect ICT.

Trabbic-Carlson et al. (2003) further elucidated the dependence of ELP sequence and MW on the properties of ELP hydrogels by designing ELPs consisting of Val-Pro-Gly-Xaa-Gly repeats, where Xaa was chosen to be Lys every 7 or 17 pentapeptides and Val at other Xaa positions. These ELPs were chemically crosslinked with tris-succinimidyl amino-triacetate to produce hydrogels. Swelling experiments indicated that hydrogel mass decreased by 80 -90% gradually over a 50°C temperature range. Gels ranged in stiffness from 1.6 to 15 kPa at 37°C depending on protein concentration, lysine content, and MW. The dynamic shear

stiffness of these gels were two orders of magnitude greater than that of non-crosslinked ELP coacervates, a finding that showed the potential for covalently-stabilized ELP scaffolds to serve as functional constructs for tissue engineering.

Chilkoti et al., (2002) have reviewed the recombinant DNA methods for the design and synthesis of ELPs for targeted delivery of radionuclides, chemotherapeutics and biomolecular therapeutics to tumors and concluded that ELPs as drug carriers enhance the localization of ELP-drug conjugates within solid tumors when external hyperthermia is applied.

MacEwan and Chilkoti., (2012) have described a newer digital technique for specific cellular uptake application of a diblock copolymer of ELP and its genetically encoded self-assembled temperature triggered CPP-arginine analog. They developed a nanoparticle micelle by modulation of diblock copolymer ELPs with the CPP-arginine. The study results revealed that temperature triggered arginine analog of ELP has increases cellular uptake when compared to the same diblock copolymer of ELPs without arginine.

Callahan et al., (2012) reported that the first pH-responsive polypeptide micelle that dissociates at the low extracellular pH of the solid tumors. They developed a histidine-rich ELP block copolymer, which self-assembles at 37°C into spherical micelles for better in vivo penetration and distribution of ELPs in tumors.

Nitta and Numata et al., (2013) have reviewed ELP-based nanoparticles for drug/gene delivery and tissue engineering applications. They observed a well-defined unique phase transition behavior of ELPs that promotes recombinant expression, protein purification and self-assembly of nanostructures. They concluded that the multi-functional biopolymer-based and biopolymer-synthetic polymer conjugated nanoparticles, precisely targets drug/gene to specific site in cancer chemotherapy.

Cho et al., (2016) demonstrated the application of immune-tolerant elastin-like polypeptides (iTEPs) as cytotoxic T-lymphocyte vaccine carriers. iTEP design is known to place an emphasis on humoral tolerance due to the absence of epitopes that can bind T and B cells. The motifs were picked to create the repeats of iTEPs A to D, they were sourced from the homologous peptide sequences of mouse and human elastin.

Fletcher and Dandan et al., (2018) reported that the ITC purification method on a larger production scale to equip the pharmaceutical biotechnological industries to use this method for purification instead of the complex, time consuming and expensive chromatography methodology, while maintaining a laboratory environment. Their methodology is suitable for soluble recombinant ELP fusion proteins. It uses the phase transition behavior of an ELP tag to separate the fusion protein from cellular contaminants. Recent studies have been made in the field of ITC technology, because conventional ITC technology needs chemical reactions or protease to remove ELP tags, and researchers have developed an indirect ITC technology using chemical modification or genetic engineering, the captured molecules that can bind specifically to the target protein are connected to ELP, thus, in the cell lysate, this recyclable ELP- captured molecules can selectively bind to the target protein, and then the routine ITC separation program can separate the target protein from the cell lysate.

Fletcher Emmanuella E et al., (2018) discussed about the existence of the new and effective methodology, inverse transition cycle coupled with elastin-like polypeptides. since they have opened the gateways into biochemical scientific research as well as a key scope in the biotechnological world.

Saxena et al., (2015) extensively studied the biodegradability of ELPs in a variety of physiological micro-environments. The rate of biodegradation controls the accumulation of ELPs at the target site besides clearance from the body and suggested a comprehensive mechanism for the degradation of ELPs. ELPs are internalized by cells pinocytosis, localized in low pH compartments, namely lysosomes and are eliminated over a period of 2–24 h after proteolytic cleavage by elastase and collagenase endopeptidases.

Bellingham et al., (2001) synthesized a family of human recombinant elastin-like polypeptides (ELPs) that have been shown to have similar phase transition and self-assembly properties as native tropoelastin. This family of ELPs of varying lengths and amino acid sequence are based on exons 20, 21, 23 and 24 of human elastin. They were characterized as having hydrophobic domains (encoded by exons 20 and 24) which are separated by hydrophilic crosslinking domains (encoded by exons 21 and 23). They have also demonstrated that when coated onto polymer surfaces, ELPs were shown to promote a low thrombogenic response when in contact with blood components.

Urry et al., (1997) demonstrated that these hydrophobic associations are responsible for a shift from complete solubility in pure water at 20 °C to a state of phase separation at 40°C with a dynamic structured state of 63% water/37% polypeptide by weight at physiological temperatures. The self-assembled form is subsequently denatured at 70°C to a disordered state consisting of 32% water/68% polypeptide. This phase separation phenomena produces a distinct relationship between the hydrophobicity of an ELP and its transition temperature. They maintain that every interaction and/or modification of which a polymer is capable can be characterized as a function of its effect on Tt. The higher the molecular weight or hydrophobicity of an ELP, such as the amino acid-based hydrophobicity scale, the lower the energy required for surrounding water molecules to enter the bulk water and thus the lower the heat required to induce hydrophobic assembly. ELPs with high molecular weights and/or hydrophobic guest residues exhibit lower transition temperatures than ELPs with low molecular weights and/or hydrophilic guest residues.

Schellenberger et al., (2009) studied that a fusion of exenatide, a therapeutic analogue of glucagon-like peptide 1 improving glycemic control, to the recombinant polypeptide known as XTEN which are related to the diabetes treatment. Based on allometric scaling in various animal models, this fusion protein was extrapolated to enhance the peptide's plasma half-life in humans from 2.4 h to 139 h, which could potentially enable once monthly administration. These results provided suitable grounds for ELP experts to expand into new lines of investigation. More specifically, the 30 amino acid glucagon-like peptide 1 (GLP-1), which possesses a short half-life, was chosen as an ELP fusion candidate due to its role in promoting insulin release from pancreatic β-cells.

C. Vepari et al., (2007) studied that Silk-like proteins (SLPs) are another type of recombinant material with demonstrated success in biomedical applications. These biopolymers are designed taking into account the repetitive peptide sequences found in silkworm and spider silk. The most common of all silk variants is probably the hexapeptide GAGAGS from Bombyx mori fibroin, although recombinant spider silk has also been used in nanoparticles for gene and drug delivery. In aqueous solution, these silk-derived polymers undergo an essentially irreversible conformation transition from random coil to beta sheet and a subsequent beta sheet aggregation growth accelerated by an increase in

temperature. The good biocompatibility and biodegradability shown by these materials, and particularly the mechanical strength of the resulting aggregation products, stimulated the design of chimeric materials such as silk elastin like recombinamers (SELRs).

CHAPTER 3

REVIEW METHODS

3. REVIEW METHODS

For the present review, at first many research paper and other scientific journals were selected related to the topic– biotechnological application of ELPs and the inverse transition cycle of ELPs. In this regard, published research article, reference book and authentic web-based information were considered. I read more than 25 full articles and a total of 60 peer-reviewed articles which focused on inverse transition cycle of ELPs and biotechnological & biomedical applications of ELPs.

Then, various information related to the topics and explanation on the effect of ELPs were studied. Following the investigation of the reported information, the review focused on protein purification by inverse transition cycle and discuss them at future perspectives. During this review information were collected from different sources. These are:

Library work

Extensive library works have been done during this review. The libraries are Khulna University Library, Seminar Library of Biotechnology and Genetic Engineering Discipline, Khulna University. The related books which are studied for this review were Biochemistry of Elastin, Biomedical engineering, Cellular Biology, Molecular Biology and Genetics. Those books relevant to the review were studies intensively for understanding the articles.

Journals and thesis paper

Scientific journals on ELPs Biochemistry are very important source for the fulfilment of this review.

Website visited

http://proteinatlas.org

https://www.pureprotein.com

http://bbcscience.com

http://sciencedaily.com

http://ncbi.nlm.nih.gov

CHAPTER 4

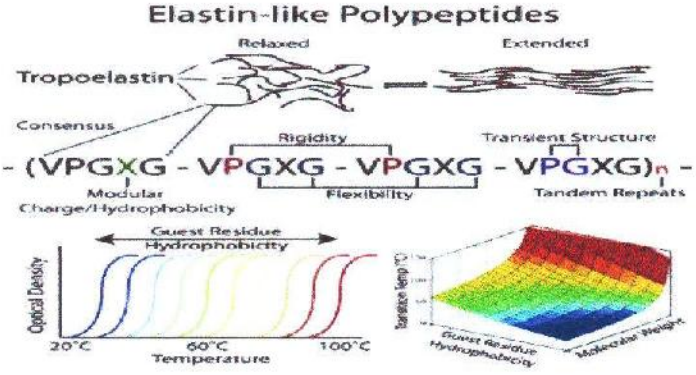
DISCUSSION

OF THE METHODOLOGY

4. ELASTIN LIKE POLYPEPTIDES

Elastin is a structural protein of the extracellular matrix present in the connective tissue of all vertebrates. Its main function is to provide elasticity for large blood vessels (aorta), ligaments, lungs and skin. Elastin is naturally composed in the hydrophobic domain of a human protein: tropoelastin, (M=60-72 kDa), a soluble precursor of elastin and plays an important role in the process of tropoelastin creation (Mithieux et al., 2005). Elastin-like polypeptides (ELPs) are synthethic biopolymers and can be emphasized as repetitive polypeptides composed of multiple replicates of pentameric motif Val-Pro-Gly-Xaa-Gly, (VPGXG) where Xaa is the guest residue, which can be replaced by any amino acid except Proline (Hassouneh et al., 2010).

One of the families of protein polymers obtained through genetic engineering attracting growing attention and becoming the subject of interest of many research teams around the world is elastin-like polypeptides. ELPs are members of a broader class of stimulus responsive ''smart'' polymers that exhibit inverse temperature phase transition behavior in response to changes in their solution environment (chilkoti et al., 2009). They are soluble at temperatures below a characteristic cloud point temperature (Tt) (also known as the inverse transition temperature) and aggregate into micron scale coacervates above the Tt (Urry et al., 1997). ELP fusion proteins provides a simple and convenient methodology for protein purification that has many advantages over current chromatographic techniques used to isolate recombinant protein.

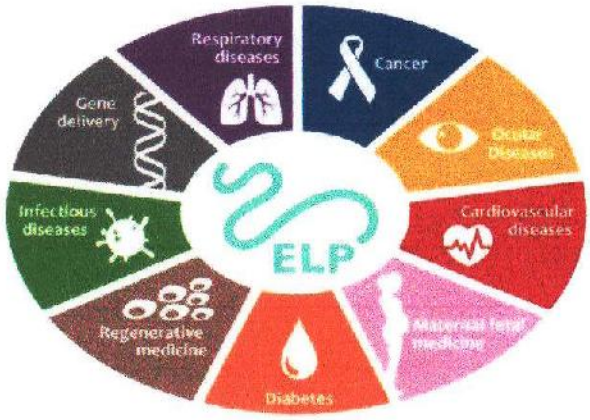


Source: https://doi.org/10.1039/C9ME00002J

Figure 1: Elastin Like Polypeptides as Building motifs.

4.1 Applications of ELPs

ELPs have many advantages over synthetic polymers. They are temperature sensitive biopolymers and undergo an inverse temperature transition (ITT) behavior in response to temperature change (Urry, 1992; Urry, 1997; Li, 2001). Biological applications of ELPs as triggered molecular actuators for drug delivery and recombinant protein purification, and as stimuli-responsive materials for tissue engineering. Many types of application of ELPs are present. These are–



Source: http://dx.doi.org/10.1016/j.jconrel.2015.11.010

Figure 2: The various applications of ELPs as nanomedicines.

Protein purification are the main application of ELP. ELPs are also useful for a wide variety of several biomedical applications for tissue engineering, drug delivery, vaccine carriers, anticancer treatment.

4.1.1 Applications of ELPs in biomedical

The versatility of ELPs as stimulus responsive biomaterials has led to their use in a variety of physiological applications. The intended application of the protein ELP fusion dictates the ELP design. Considerations such as molecular weight, transition temperature, and self-assembly must be considered in choosing the number of pentapeptide repeats, the guest

residue composition, and biopolymer architecture. Use of an ELP solely as a soluble macromolecular carrier provides advantages of improved pharmacokinetics and increased plasma half-life of the therapeutic fusion. For these ELPs, which are typically single segment polymers, the T_t should be designed to be sufficiently above body temperature to prevent aggregation at physiological conditions. Single segment ELP can also be used to provide a gel-like depot for prolonged local release of a therapeutic ELP fusion. Depot-forming ELPs require careful tuning of the T_t above the temperature at which they can be injected in their soluble form (for instance, at room temperature), and below the body temperature at which they should aggregate. ELPs can also be assembled into nano-scale structures by design of ELP block copolymers whose unique hydrophilic and hydrophobic domains maintain independent transition temperatures (Dreher, 2008; MacKay, 2009). Such ELP nanoparticles may improve systemic pharmacokinetics and enhance targeting to some disease sites such as solid tumors. The amino acid composition and molecular weight of hydrophobic and hydrophilic ELP domains will dictate the critical micelle temperature (CMT), influence micelle stability over the intended temperature range, and determine the micelle's hydrodynamic radius. By choice of ELP sequence and size, the CMT can be tuned well below body temperature, such that the ELP exists in a micellar form at all physiological thermal conditions. Alternatively, the CMT can be tuned to provide micelle assembly in response to a thermal trigger, such that heating of a disease target *in vivo* (at temperatures achieved by mild clinical hyperthermia, for instance) permits *in situ* self-assembly of ELP micelles only within the targeted tissue. This approach can be used, for example, to manipulate the density of peptide ligands presented on the micelle corona in order to improve local accumulation by high avidity interactions only within heated tissues (Simnick et al., 2010).

Proteins are increasingly attractive as therapeutics for the treatment of a variety of diseases due to their high specificity and activity. The kinetics of biodegradation have also been analyzed in vivo using a 59.4 kDa 14C-labeled ELP. In that study, intravenous administration led to a relatively modest degradation rate of ~2.5 wt% per day, pointing to the fact that ELPs can achieve in vivo stability for therapeutic applications (Liu et al., 2006).

ELPs play a role in temperature-triggered depot formation in tumors by the application of external hyperthermia. Recent studies have shown that tumor retention correlates with a decrease in the ELP Tt (macEwan et al., 2004). Also, some ELP fusion proteins (specifically, the IFN-ELP and the RGD-TRAIL-ELP fusion proteins) are long acting and highly potent for cancer therapy (Sarangthem & Hu., 2013). In addition, novel ELP-based solutions have been created to combat corneal wounds and Ocular diseases (Despanie et al., 2016). ELPs have also been discovered to play a major role in elevating the half-life of protein drugs in vivo.

4.1.2 ELP depots for applications in Protein Purification

Protein purification began over two centuries ago. Several improved chromatography procedures have evolved to purify recombinant proteins at bench-scale over the last few years (Guan et al., 2014), significantly, the immobilized metal ion affinity chromatography (IMAC). IMAC has been reported as a known powerful method for protein purification but it has limitations. It requires costly resins and some native proteins present in the cell lysate have sufficient affinity for metal ions to bind to the IMAC resin, which typically reduces the level of purification achieved by IMAC (Hassouneh et al., 2010). Presently, highly pure proteins are being analyzed in research, for use as reagents in biotechnology and medicine as well as therapeutics. These scientific and economic necessities have provided the motive for the researchers to develop further improved methods to purify proteins that are productive, flexible, stable and cost effective. The existence of the new and effective methodology, inverse transition cycle coupled with elastin-like polypeptides have been discussed in this review since they have opened the gateways into biochemical scientific research as well as a key scope in the biotechnological world.

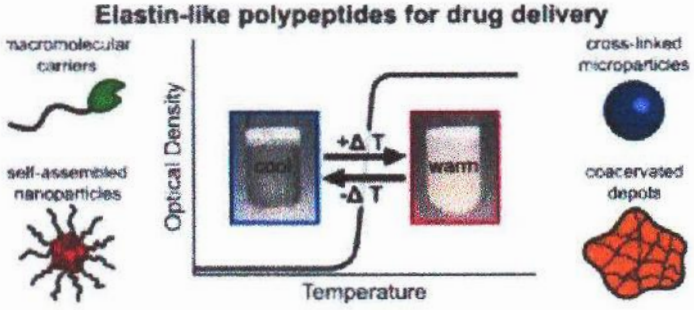
ELPs mainly aid in protein purification, they are usually fused to other proteins before purification. The stimulus responsiveness of ELP fusion proteins provides a simple and convenient methodology for protein purification that has many advantages over current chromatographic techniques used to isolate recombinant proteins (chilkoti et al., 2009). Single type of protein can be isolated from a complex mixture by protein purification method.

Protein purification has a variety of applications in drug delivery, tissue engineering, and bio interface science. Most advantages of this technique are the low cost of purification. This technique is very simply which that requirement only a laboratory centrifuge or filter that is readily available in most molecular biology and biochemistry laboratories. Other advantages are ease of multiplexing, and high yield. purified proteins are important for many biomedical applications such as therapeutics and diagnostics, and in regenerative medicine and biosensing. Also, these purified proteins are an important factor in drug discovery.

4.1.3 Assemblies of ELP drug carriers

ELPs are known to be vehicles for drug delivery. ELPs are biocompatible and are therefore suitable for local and systemic administration, as they induce minimal inflammatory and immune effects in animal models (Urry et al., 1991) and can be administered to humans without eliciting an adverse immune response (Christiansen et al., 2013).

Many cancer therapeutics have limited efficacy because of severe systemic toxicity or suboptimal pharmacokinetics within the body (Satchi-Fainaro et al., 2006).



Source: https://doi.org/10.1016/j.jconrel.2014.06.028

Figure 3: ELPs for drug delivery system

Table-1: ELP-functionalized hybrid drug delivery systems

Application	ELP sequence	Functionalization
ELP-functionalized plasmonic nanoparticles	$(VPGXG)_{180}$ X= $V_5G_3A_2$	Lysine residue (and N-terminal amine) for electrostatic adsorption or covalent grafting to MUA functionalized gold nanoparticles
	$[VG-(VPGVG)_4-VPG]_8-[VG-(VPGVG)_2-VPGCG-VPGVG-VPG]_2$	Cysteine residues for functionalization of gold nanorods through gold thiol bonds
	$[VG-(VPGVG)_4-VPG]_8-[VG-(VPGVG)_2-VPGCG-VPGVG-VPG]_8$ or 12	Cysteine residues for functionalization of gold nanorods and cross-linking of composite matrices with physical encapsulation of 17-AAG drug
ELP-functionalized liposomes	$(VPGVG)_3$	Stearyl group functionalization for incorporation into bilayer of doxorubicin-encapsulating PEGylated liposomes and cyclic RGD-decorated doxorubicin encapsulating PEGylated liposomes
ELP-functionalized dendrimers	$(VPGVG)_1$	Boc-functionalization for conjugation of ELP C-terminus to amines on PAMAM dendrimers with physical encapsulation of model drug rose Bengal

Source: doi: 10.1016/j.jconrel.2014.06.028

4.1.4 ELP depots for applications in Tissue Engineering

ELP is a promising polymer that can be used for tissue engineering and highly customizable scaffold. ELPs are used for tissue engineering owing to their customizable physical properties and biocompatibility. ELP can be applied to tissue engineering, not only because it has good biocompatibility, biodegradability, and non-immunogenicity, but also because it can accurately control its amino acid sequence, chain length, polymer structure and type, quantity and crosslinking position at the gene level and synthesis level, so that it can be refined (Hassouneh et al., 2012). These types of properties are more attractive for in vivo applications such as tissue engineering scaffolds. These attributes of

ELPs have led to their use in tissue engineering and regenerative medicine for cartilage and intervertebral disc tissue repair, small-diameter vascular grafts, urinary bladders, stem cell matrices, neural guides, post-surgical wound treatment, and generation of stem cell sheets (Arias et al., 2006).

The fine control over ELP sequence and length allows precise manipulation of their biological activity, chemical reactivity, and physical properties, including their stimulus-responsive and self-assembly behavior, making them attractive for biomedical applications. In this review, focused on the design and synthesis of ELPs, ELP fusions, and ELP-based materials in which the mechanical and biochemical properties of ELPs are enhanced for tissue engineering and drug delivery applications.

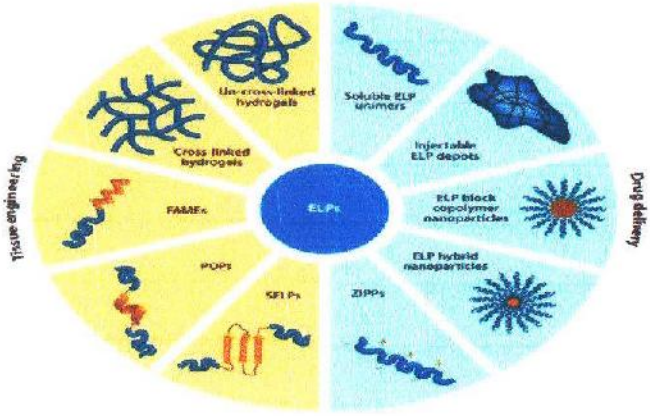


Figure 4: ELP-based materials that have been engineered for tissue engineering and drug delivery applications.

FAME, fatty acid–modified ELP; POP, partially ordered polymer; SELP, silk ELP; ZIPP, zwitterionic polypeptide.

4.1.5 ELP depots for applications in Vaccine Carriers

The application of immune-tolerant elastin-like polypeptides (iTEPs) as cytotoxic T-lymphocyte vaccine carriers. iTEP design is known to place an emphasis on humoral tolerance due to the absence of epitopes that can bind T and B cells (Cho et al., 2016). The

motifs were picked to create the repeats of iTEPs A to D, they were sourced from the homologous peptide sequences of mouse and human elastin. Although lacking the canonical pentapeptide sequences, iTEPs still maintain phase transition properties. Each of the four iTEPs produced minor iTEP-specific antibody titers relative to an ovalbumin control. One more remarkable finding was the fact that in vivo aggregation of hydrophobic constructs (iTEPs A, C, and D) did not impact immunogenicity in any manner differing from soluble forms (iTEPs B) (Despanie et al., 2016).

4.1.6 ELP depots for applications in Anticancer Drug

Cancer treatment is a multi-pronged strategy. ELPs plays an important role in cancer treatment. One of the main problems of modern oncology is often the lack of selective carriers of antineoplastic drugs, delivering the drug only to pathologically changed tissue. In most cases, only a small portion of the drug reaches its destination, while the remaining part has a negative influence on healthy cells. This makes it necessary to develop carriers that will make chemotherapy less toxic and more effective. Thermally responsive elastin-like polypeptides (ELPs) have also been examined. These polymers have several attractive features. In particular their ITT behavior has attracted the attention of researches. The tunable ITT of ELPs between 37°C and 42°C can be exploited for delivering drugs to solid tumors by the application of external hyperthermia. As temperature increases above ITT, ELPs undergo reversible phase transition and form insoluble aggregates and are internalized by cells via pinocytosis. Mild hyperthermia promotes high blood flow and high vascular permeability of therapeutic agents at tumor site (Fujiwara & Watanabe., 1990).

Current research into neoplasm therapy already uses synthetic and natural soluble polymers such as liposomes (Sofou., 2010; Cai., 2014). One of the first cancer-based applications was performed by Dr. Chilkoti and colleagues, who demonstrated that hyperthermia could enhance tumor localization of ELPs in SKOV-3 ovarian carcinoma and D54MG glioma cell lines Several anticancer-drug conjugates have entered into clinical development, and a growing number of polymeric drugs and PEGylated proteins have since come into the market (Duncan and Vicent, 2013). They observed aggregates of the thermally responsive ELP within the heated tumor and concluded that accumulation is caused due to its ITT behavior. Further, the results of studies also reveal the preferential accumulation of the

thermally responsive ELPs in heated tumors due to the combined effect of EPR and hyperthermia. ELPs as carriers enhance the efficacy of antitumor therapeutics when compared to the administration of free drugs by three mechanisms, namely increasing the overall accumulation within solid tumors, providing a homogeneous spatial distribution in tumor tissues and increasing the intracellular localization of anticancer therapeutics. Meyer et al., (2001) have also performed a study using a genetically engineered ELP and synthetic thermally responsive copolymer of N-isopropylacrylamide (NIPAAm) and showed a 2-fold increase in the accumulation of drug in ovarian tumors implanted in dorsal skin of nude mice with local hyperthermia. The results thus reveal that enhanced delivery of anticancer drugs to solid tumors can be achieved by conjugating drugs to thermally responsive natural as well as synthetic polymers with mild hyperthermia. The recombinant DNA methods for the design and synthesis of ELPs for targeted delivery of radionuclides, chemotherapeutics and biomolecular therapeutics to tumors and concluded that ELPs as drug carriers enhance the localization of ELP-drug conjugates within solid tumors when external hyperthermia is applied (Chilkoti et al., (2002). They have also studied that the results on the development of macromolecular carriers for thermal targeting of therapeutics to solid tumors. The two thermally responsive polymers namely, poly(N-isopropylacrylamide-coacrylamide) [poly (NIPAAm)] and an artificial ELP designed by them exhibited ITT behavior at a temperature slightly >37°C. In vivo fluorescent videomicroscopy and radiolabel distribution studies of ELP delivery to human tumors implanted in nude mice demonstrated that hyperthermic targeting of the thermally responsive ELP for 1 h provides a 2-fold increase in tumor localization compared to the same polypeptide without hyperthermia. Similar results were also obtained for poly (NIPAAm) though the extent of accumulation was somewhat lesser than observed for ELP. A thermo-responsive and genetically engineered ELP containing a C-terminal cysteine residue and conjugated Doxorubicin to the ELP through different pH-sensitive and maleimide activated hydrazone linkers. The results revealed that the ELP-Doxorubicin conjugates with longer linkers exhibit slower drug release kinetics compared to the ELP-Doxorubicin conjugates with shorter linkers (Furgeson et al., 2006).

Table 2: Therapeutic ELP depots of Cancer & Diabetes.

Application	ELP sequence	Functionalization
Depots for cancer	$(VPGVG)_{120}$	Tyrosine residue for conjugation of ^{125}I or ^{131}I radionuclides.
	$(VPGVG)_{60,\ 120,\ or\ 240}$	1, 4, or 7 tyrosine residues for conjugation of ^{125}I or ^{131}I radionuclides.
	$(VPGXG)_{160}$ or 168 X=$A_{14}V1C1$ or V_aC_b where a:b=15:1, 9:1, or 5:1	Cysteine residues for disulfide cross-linking and tyrosin residues for conjugation of ^{125}I radionucleotide.
Depots for diabetes	$(VPGVG)_{120}$	GLP-1 fusion for release of macromolecular peptide drug carrier.
	$(VPGVG)_{240}$	(GLP-1)6 fusion via protease cleavable arginine linkers for release of free peptide drug.

Source: doi: 10.1016/j.jconrel.2014.06.028

4.1.7 ELP depots for applications in Diabetes

Diabetes describes a cluster of chronic metabolic diseases, involving high blood glucose levels and insulin insufficiency, which affects more than 300 million people worldwide; the CDC further predicts that a staggering 1 in 3 Americans could be stricken by 2050 (C.f.D.C.a Prevention, 2010). Within this patient population, type 2 diabetes essentially amounts to 90 to 95% of all cases diagnosed in adults. Fusion of exenatide, a therapeutic analogue of glucagon-like peptide 1 improving glycemic control, to the recombinant polypeptide known as XTEN (Schellenberger et al., 2009). Based on allometric scaling in various animal models, this fusion protein was extrapolated to enhance the peptide's plasma half-life in humans from 2.4 h to 139 h, which could potentially enable once monthly administration. These results provided suitable grounds for ELP experts to expand into new lines of investigation. More specifically, the 30 amino acid glucagon-like peptide 1 (GLP-1), which possesses a short half-life, was chosen as an ELP fusion candidate due to its role in promoting insulin release from pancreatic β-cells (Amiram et al., 2013). Two 50 kDa (GLP1)-ELPs were designed with the first possessing room temperature solubility and the ability to form a stable, coacervate-based drug depot following subcutaneous injection into

C57BL/6J mice; the second fusion served as a control which remained soluble at body temperature. Additional work has been conducted with a proinsulin-ELP formulation, capable of being converted into mature insulin following subcutaneous administration, alongside novel coformulations involving (GLP-1).

Table 3: Elastin like polypeptide nanomedicines under commercial investigation.

Product/Agent	Indications	Status	Company
siEVI1-ELP	Various cancers (breast, ovarian, pancreatic, and lung)	Preclinical	PeptiMed
Liposome-ELP conjugates	Breast cancer	Preclinical	Samsung
VIP-ELP (Vasomera™)	Pulmonary arterial hypertension, cardiomyopathies, cystic fibrosis	Phase I	PhaseBio
GLP1-ELP (Glymera™)	Type II diabetes	Phase IIB	PhaseBio

Source: http://dx.doi.org/10.1016/j.jconrel.2015.11.010

4.1.8 ELP depots for applications Cardio-vascular diseases

Cardiovascular diseases (CVDs) mark the primary cause of death worldwide with 600,000 fatalities reported each year in the US alone. Similarly sobering is the fact that CVD accounts for 313 billion USD in costs due to health expenditures and lost productivity (Healthline., 2014). Among the risk factors for CVD, hypertension remains most amenable to pharmacological modulation as indicated by the broad range of antihypertensive medications on the market. PhaseBio Inc. has pioneered an ELP-based treatment for essential hypertension using a VIP-ELP fusion protein known as Vasomera™. Vasoactive intestinal peptide (VIP) is a neuropeptide, consisting of 28 amino acid residues, which acts

as a ligand of the G-protein coupled receptors VPAC1 and VPAC2 (Dvorakova et al., 2014). As VIP promotes heart contractility and induces coronary vasodilation, therapeutic applications against hypertension provide a compelling rationale for its exploitation.

VIP-ELP was subsequently investigated for its potential in mitigating heart failure using rat models of doxorubicin-induced cardiomyopathy (Rio et al., 2012). Daily intravenous administration of VIP-ELPs combated myocardial dysfunction and prevented muscle wasting. These protein polymers also reduced the energetic demands of diseased cardiac tissue while simultaneously improving left ventricular systolic/diastolic functioning. When canines were given the VIP-ELPs, both groups with healthy and failing hearts exhibited a dose-dependent increases in cardiac function within the 0.1 to 1 μg/kg/min range (Rio et al., 2013). Those precedents established the foundation for Phase I single ascending dose trials by PhaseBio in patients with pulmonary arterial hypertension. Introduced through once weekly dosing regimens, VIP-ELPs were found to be safe and well-tolerated by subcutaneous and intravenous routes. They also demonstrated a prolonged, dose-dependent reduction in blood pressure. Recent work now focuses on treating the cardiac dysfunction associated with both Duchenne and Becker muscular dystrophy (Pharmaceutics et al, 2013).

4.1.9 ELP depots for applications Ocular diseases

With the National Eye Institute reporting an economic burden for eye diseases surpassing 139 billion USD in 2014, ocular therapeutics represent another urgent growth opportunity. ELP-based solutions have been devised to combat corneal wounds and Sjögren's syndrome (Haddadin et al., 2013). The autoimmune disease known as Sjögren's syndrome (SjS) affects approximately 4 million patients with 9 out of 10 being women (Patel et al., 2014). The primary symptoms include debilitating dry eye and dry mouth. The MacKay group, in collaboration with the Hamm-Alvarez laboratory, therefore sought to further adapt the FSI rapamycin nano-formulation discussed earlier to encompass ocular indications (Shah et al., 2013). This rationale arises from the fact that, while this small molecule drug acts as an immunosuppressant, it has never been investigated as a means of treating the severe

autoimmune inflammation of the lacrimal gland, called dacryoadenitis, using the NOD model that replicates some of the pathology found in SjS.

4.1.10 Other Applications of ELP

ELPs are useful for a wide variety of biomedical applications for several reasons.

There are-

1. The LCST of ELPs can be precisely tuned between 0–100°C with a precision of a few degrees Celsius. This allows for the design of ELPs that are optimized for a specific application (e.g., drug delivery, protein purification, etc.).

2. ELPs can be synthesized as monodisperse polymers in *E. coli* from synthetic genes. The precisely-defined MW of ELP is ideal for drug delivery because MW is the key parameter that controls both the route of clearance from the body and the in vivo half-life of a polymer.

3. ELPs can be expressed at gram per liter quantities in a laboratory setting, allowing ELP production at a cost that rivals that of synthetic polymers.

4. The thermal responsiveness of ELPs permits their purification without complex chromatographic separation simply by exploiting the phase transition behavior of ELPs.

5. ELPs appear to be biocompatible, suggesting their suitability for in vivo applications.

4.2 Inverse Transition cycle

The inverse transition cycle methodology, the protein was successfully purified. The use of self-cleaving ELP intein tags is viable in high cell-density *E. coli* fermentation and can provide highly purified target proteins at a reasonably low cost (Fong et al., 2010). The ITC technique exploits the unique phase transition behavior of ELPs. In ITC, a peptide or target protein is fused to the ELP and expressed in *E. coli* or any other expression system and purified using the ELP as a purification tag (Meyer et al., 1999). ITC is not only inexpensive but also it is time-efficient since it omits chromatography in the purification method (Hassouneh et al., 2010).

ITC is technically simple, fast, economical, and requires no specialized equipment or reagents. The ITC purification method on a larger production scale to equip the pharmaceutical biotechnological industries to use this method for purification instead of the complex, time consuming and expensive chromatography methodology, while maintaining a laboratory environment. This methodology is suitable for soluble recombinant ELP fusion proteins. It uses the phase transition behavior of an ELP tag to separate the fusion protein from cellular contaminants (Gibson et al., 2013). The transition temperature of the protein ELP fusion is lowered below the ambient temperature when the salt concentration of the solution is increased causing aggregation of the ELP fusion protein and hence the formation of a pellet upon centrifugation of the sample ("hot spin"). Upon resuspension of the sample in a low ionic strength buffer, the ELP fusion re-dissolves, and any residual insoluble contaminants are removed by centrifugation at low temperature (typically 4°C, and thus termed "cold spin"). The supernatant from this step contains the purified ELP fusion and this process makes up one round of ITC. This procedure can be repeatedly performed to increase the purity of recombinant ELP fusion protein (McCarthy et al., 2016). Separation can be completed in minutes using standard laboratory centrifuges and NaCl. Furthermore, these advantages can be realized over a wide range of purification scales. For micro-liter-scale expression and purification, ITC can be employed in parallel for high-throughput applications. At the other extreme, because it avoids the expense associated with traditional chromatographic separations, ITC is also likely to be useful for the purification of single proteins at the gram-to-kilogram scale in industrial bioprocessing (Meyer and Chilkoti., 1999).

The number of ITC depends on the nature of different target proteins. However, there is a loss of a part of the target protein in each cycle, while increasing the purity of the protein. The following is a flow chart (Figure-3) for isolation and purification of recombinant protein by ITC. Compared with conventional column chromatography, the efficiency of ITC technology has been proven to be beneficial, inexpensive and very productive. To date, ITC has been used to successfully purify a number of different proteins (Carlson et al., 2004), and it has been demonstrated that the activity of every reported target protein is preserved with ITC. The purification of ELPs by means of their LCST behavior presents a simple and chromatography-free approach to purify the majority of ELPs and peptide or

protein ELP fusions expressed in *E. coli* (Macewan., 2014; Hassouneh., 2012). In recent years, new research achievements have been made in the field of ITC technology, because conventional ITC technology needs chemical reactions or protease to remove ELP tags, and researchers have developed an indirect ITC technology using chemical modification or genetic engineering, the captured molecules that can bind specifically to the target protein are connected to ELP, thus, in the cell lysate, this recyclable ELP-captured molecules can selectively bind to the target protein, and then the routine ITC separation program can separate the target protein from the cell lysate (Stiborova et al., 2003). ITC is also useful for the study of protein-ligand interactions and forms the basis for competitive solution immunoassays (Meyer et al., 2002).

To produce large quantities for industrial use in the pharmaceutical industries, the use of large volume and high-speed modern equipment (such as SP-3000 Ultrasonic Processor and the APD Centrifuge series) that can be controlled per the users' specifications is suggested. This enables the purification process to be hypothetically reduced to a shorter time duration as compared to the usually known laboratory setup as demonstrated by (MacEwan et al., 2014). The reality that ELPs can be genetically supplemented to its protein or peptide partner with precise control over the gene design of ELP fusion (N-or C-terminal fusion), and that the thermal properties of the ELP tag can be easily tuned to make ELP fusions attractive and advantageous for recombinant protein purification (Ge et al., 2005). Over the years ELPs have been fused to many other proteins, all these fusions have been shown to play significant roles in diverse areas of science. ELP fusion proteins have been researched by many scientists in *E. coli* cells, mammalian and plant cells as shown in table 4.

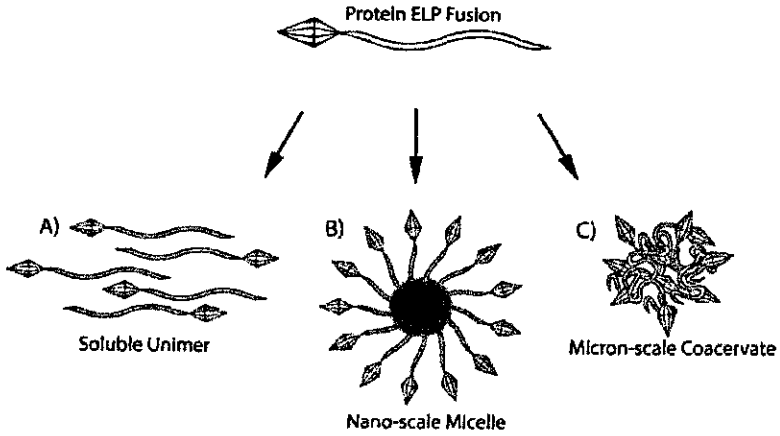
Table 4. ELP fusion proteins in various expression system.

Fusion Protein	Expression system
Mini-glycoprotein (gp 130)	Tobacco leaves
Anti-HIV-1 monoclonal antibody 2F5	Transgenic tobacco plants & Chinese hamster ovary (CHO) cells

Anti-HIV-1 antibody 2G12	Transgenic tobacco leaves & seeds
Mycobacterial antigens (TBAg)	Transgenic tobacco plants
Avian flu H5N1 antigens (2)	Transgenic tobacco plants
Spider silk elastin protein	Tobacco & potato plants
Human interleukin -10	Tobacco cell suspension culture
Tumor necrosis Factor (TNF-V(H)H -ELP)	Transgenic tobacco plants
Keratinocyte Growth Factor (KGF)	*E. coli*
antimicrobial peptide Halocidin18 (Hal18)	*E. coli*
Thioredoxin	*E. coli*
Cell penetrating peptides (such as penetratin peptide and Tat peptide)	*E. coli*
Interleukin-1 receptor antagonist	*E. coli*
KLAKLAKKLAKLAK (KLAK) peptide	*E. coli*
Glucagon-like peptide	*E. coli*
Interferon alpha (IFN) fusion protein	*E. coli*
RGD-TRAIL	*E. coli*
ELP- C2 domain of streptococcal protein G	*E. coli*

Source: http://doi.org/10.1016/j.pep.2018.09.006

is soluble at room temperature and undergoes aggregation at 37°C to form a viscous coacervate (Figure 5C). This allows ease of injection at a desired site as a soluble material, while prolonging residence of the protein ELP fusion at the target by forming an insoluble depot (Liu et al., 2010).



Source: http://dx.doi.org/10.1016/B978-0-12-416039-2.00024-0

Figure 5: Application of therapeutic protein ELP fusions.

The physiochemical properties and biocompatibility of ELPs make them a desirable material for physiological applications. With their fusion to pharmacological proteins ELPs can serve as therapeutic carriers in a variety of approaches to overcome challenges such as low solubility, fast clearance, and poor delivery that is typical to the application of protein drugs. The thermal properties of ELPs provide several means of improving the efficacy of its therapeutic protein cargo. Protein ELP fusions as soluble unimers provide improved solubility and prolonged circulation when systemically administered (A). ELP block copolymers, capable of forming spherical micelles, can enhance the avidity of interaction between proteins and their desired targets by the increased density of targeting peptides or proteins on the micelle corona (B). Alternatively, proteins can be sequestered and protected in a micelle core. By tuning of the ELP transition temperature, protein ELP fusions can be designed to aggregate at physiologic temperatures upon injection, and thereby provide an in-situ depot to prolong the residence time of the therapeutic protein ELP fusion at the desired site of action (C) to act as a depot for loco-regional therapy or as a depot for the sustained release of the ELP fusion into systemic circulation.

4.2.2 Design of ELP fusion proteins

Although ELPs can be used as purification tags for a wide range of proteins and peptides, each target protein or peptide will have unique properties that may require adjustment of the ITC purification protocol (Macewan et al., 2014). Below are briefly discussed variables that can alter the purification method.

Length of ELPs label (n repeat number)

This nomenclature is used to identify ELPs: ELP[XaYbZc-n] where X, Y, Z indicates the one letter code of each amino acid in the guest residue position, a, b, c specifies the number of amino acids in the monomer gene, and n denotes the number of pentapeptide repeats. Up till now, the most widely used ELP in fusion constructs is ELP [Val5Ala2Gly3- 90] (Carlson et al., 2004). The routine use of this construct is large because of the following reasons. The length of the ELP and its guest residue composition are two orthogonal parameters in ELP design that can be used to control the Tt, where hydrophobic guest residues and longer chain lengths result in lower Tts, while hydrophilic guest residues and shorter chain lengths result in lower Tts, while hydrophilic guest residues and shorter chain lengths result in higher Tts (Schipperus et al., 2009). ELP concentration is inversely related to the Tt, where solutions of greater ELP concentration have lower Tts. The type and concentration of salts also influence the ELP Tt, where the effect of salts follows the Hofmeister series (Cho and Zhang., 2008).

ELP genes of a desired length (typically encoding 60-180 pentapeptide repeats) require oligomerization of a synthetic gene that encodes a 'monomer' repeat, as typical chemical synthesis of DNA only yields ssDNA sequences of <150 bases in length. To speed the synthesis of long, repetitive genes initial oligomerization of several monomer gene repeats is achieved with concatemerization, in which multiple copies of small oligonucleotides can be inserted into the cloning vector in a single step. This initial insertion of multiple monomers of the repetitive gene provides little control over the length of ELP genes created and is a process typically done prior to gene oligomerization that is separate from PRe-RDL, which permits the creation of long ELP genes of precise length from the products of concatemerization. The monomer is repeat on which the ELP sequence is based can be a

single pentapeptide repeat or several pentapeptide repeats in length, depending on the desired length of the final ELP product (the total pentapeptide repeats must be a multiple of the repeats contained in the monomer unit) and the speed with which the gene encoding the final ELP product can be oligomerized by successive rounds of Pre-RDL (larger monomer repeats reduce the number of cloning steps toward a final ELP gene).

The Relative Position of ELP Tag and Target Protein (N, C)

Fusions of an ELP at the N or C terminus to a target protein at the gene level enable their easy purification by an inexpensive, non-chromatographic technique developed in the laboratory, labeled as inverse transition cycle (ITC) (VerHeul et al., 2018). This technique has been employed in many applications. The point of fusion of the target protein correlates to the expression levels and specific activity of the ELP fusion protein. The ELP tag has no fixed pattern at the C or N ends of the target protein. If the expression level of the target protein is high, the ELP tag at the N side may interfere with the correct folding of the target protein. In this case, the design of the ELP label at the target protein C end may be more appropriate, thus avoiding the interference of the ELP label on the target protein folding. If the expression level of the target protein is relatively low, the ELP tag is designed at the N end of the target protein, which will increase the soluble expression of the target protein. It is possible to assist the correct folding of the target protein through the hydrophobic interaction with the unfolded target protein chain, and to prevent the occurrence of in the intermolecular cluster during the target protein folding through the spatial steric effect of ELP (Nigel et al.,2006). An attempt to optimize ITC, characterized the expression and purification, by ITC, of fusion proteins where the ELP tag was fused to either the C- or the N-terminus of the target protein. In all 4 fusion cases reported Cho found that protein-ELP fusions (C-terminus) had a greater level of expression than ELP-protein fusions (N-terminus). They also observed that concentrations of all ELP-protein fusions were lower in the soluble cell lysate as compared to protein-ELP fusions (Christensen et al., 2009). Furthermore, these differences were intensified when the metric used for evaluation was functionally active rather than protein concentration, as three out of four ELP-protein fusions had considerably lower specific activity as compared to their corresponding

protein-ELP construct. For obvious reasons, purification labels are preferably placed away from protein binding regions as far as possible. If the binding site is far away from the N-end or the C-end, it can be at any end. The position of the target protein relative to the ELP tag in ELP fusion proteins is an important factor in controlling the expression level and specific activity of ELP fusion proteins. When the ELP tag was fused with protein at the N-terminus, higher expression levels and specific activity levels were shown as compared to when the ELP tag was fused at the C- terminus, which revealed low expression levels. If a specific bio-drug protein has its binding epitopes at or near the C end, this preferred one-way positioning of the ELP purification label may be a limiting factor and needs further study. Therefore, standardization of localization of ELP tags based on the nature of target proteins may need further development to permit the use of this technology to purify large numbers of biomolecules.

Thermal conditions

The thermal properties of each ELP depend on parameters intrinsic to the biopolymer such as its molecular weight (MW) and sequence, as well as extrinsic factors including its concentration in solution and the presence of other co-solutes, such as salts typically NaCl (1-3 M), ammonium sulfate (0.4-1 M) or sodium citrate (0.15-0.5 M).

4.2.3 ELPs stability

The solvent isotope effects on phase transition temperature of various neutral ELPs. The LCST of five neutral ELPs, ELP V5-120, ELP QV6-112, ELP V5A2G3-330, ELP V5A2G3-120, and ELP V5A2G3-60 were measured in light and heavy water. Secondary structure of the ELPs was quantified by amide I band Fourier transform infrared (FTIR) spectroscopy FTIR, and circular dichroism (CD) spectroscopy and change in enthalpy upon hydrophobic collapse were measured by differential scanning calorimetry (DSC) (cho et al., 2009). Their data strongly suggested that the hydrogen bonding is an important factor in stabilization of the ELP. The results of this study implied that the hydrogen bonding can

be a major factor in stability of biomacromolecules rather than hydrophobicity. ELPs are stable over long periods of time in various conditions, since the side chain for the Gly (G), Val (V), and Pro (P) residues are either simply the hydrogen atom or an aliphatic grouping (Harry et al., 2013). A collagen-like polypeptide (CLP) fused to an elastin-like polypeptide (ELP) and the CLP-ELP tag was used to make recombinant fusion protein. CLP-ELP was fused to superoxide dismutase (SOD) and D-amino acid oxidase (DAAO) with improved stability (Liu et al., 2018). The intrusive effect of H_2O_2 on the secondary structures of the recombinant enzymes was largely reduced. The stability of the recombinant enzymes against denaturing by urea was improved. ELP fusion proteins are popularly known for increased half-life and its long–term efficacy. About 20 fusion proteins have been constructed strictly for extension of half-life (Strohl et al., 2015). They demonstrated that fusion of ELP sequences to small therapeutic proteins enhances the half-life of those proteins by giving them a larger hydrodynamic radius and thus they are not eliminated by the kidney. An antitumor necrosis factor (TNF)-α VHH (single-domain) antibody of about 12 kDa was fused with ELP, resulting in a substantially increased half-life of the VHH protein in rodent models. The immobilization of the enzyme could improve the stability and tolerance of the enzyme (Zhou et al., 2017). Recently, we constructed Glu (β-glucosidase)-linker-ELP-GB (GEH) system containing binary tags, ELP and His-tag. Our data showed the ELP label did not affect enzyme activity and secondary structure of the Glu, but also significantly improved the thermal stability of the Glu. The stability of the secondary structure of fusion Glu was higher than that of Glu under the treatment of protein denaturant reagent (guanidine hydrochloride and urea).

4.2.4 Expression and extraction of protein ELP fusions

This section is described the conditions to grow and express protein ELP fusions.

1.Transform expression plasmid with inserted gene of protein ELP fusion into an *E. coli* expression strain.

↓

2.Grow single colony in 3 mL of autoclaved TB culture at 37°C overnight while shaking at 225 rpm.

↓

3.Add 1 mL of overnight culture from step 2 to an autoclaved 1L TB culture in a 4L flask. Add appropriate volume of antibiotic to culture. Incubate at 37 °C while shaking at

↓

4.Transfer culture into a 1 L bottle and centrifuge at 16000×*g* for 15 min at 4°C.

↓

5.Decant supernatant and resuspend pellet in PBS to a volume of 45 mL and transfer to a 50 mL conical tube.

↓

6.Sonicate cells while on ice at 85 W for 9 min in cycles of 10 sec on and 20 sec off.

↓

7.Add 2 mL of 10% (v/v) Poly-ethylamine (PEI) to the sonicated cell mixture.

↓

8.Centrifuge the mixture from step 7 at 16,000×*g* for 10 min at 4°C.

↓

9.Transfer supernatant to a clean tube(s). Add salt to the supernatant step-wise until the solution turns turbid. The most common choice of salt is NaCl, which can be added at a concentration of up to 3 M.

↓

10.Centrifuge the sample at 16,000×g for 10 min at room temperature.

↓

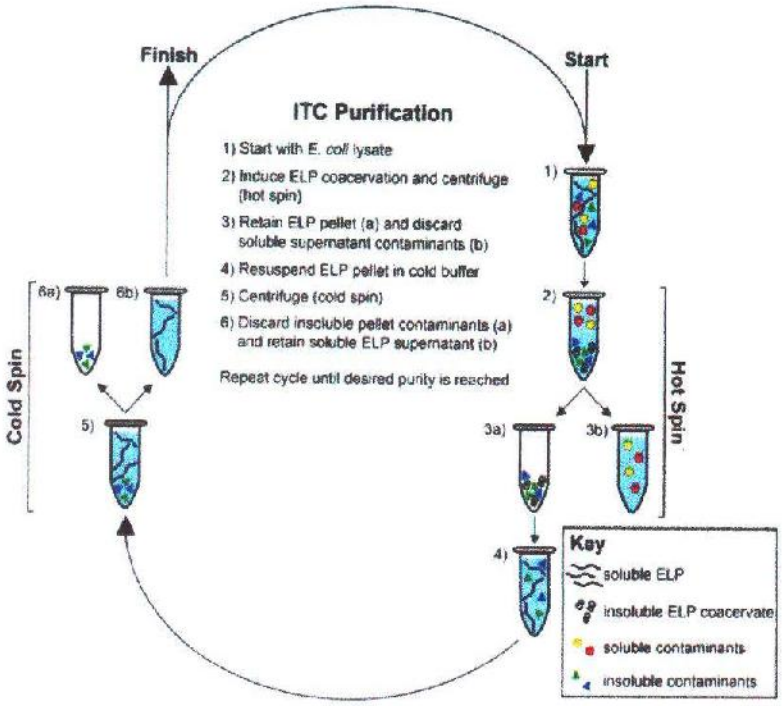
11.Decant supernatant. Add 3-4 mL of PBS (or chosen purification buffer) to pellet and resuspend by pipetting solution.

↓

12.Once the pellet is completely resuspended, transfer solution into 1.5 mL centrifuge tubes.

4.3 Inverse transition cycling

ITC purification is a convenient method for a variety of separations. ITC reduces the amount of soluble and insoluble contaminants. An ITC consists of one "cold spin" and one "hot spin", which simply refer to the relative temperature at which the centrifugation steps are performed. The cold spin is carried out under conditions of salt and temperature at which the protein ELP fusion is soluble, and it removes contaminants that are insoluble from the solution. The hot spin, in contrast, is carried out under conditions of salt and temperature at which the ELP fusion protein is in its phase separated coacervated state (Hassouneh et al., 2012) . This step separates the coacervated protein ELP fusions from other soluble molecules in solution, thus leaving the soluble contaminants in the supernatant. Reduced amounts of salts should be added in each subsequent round of ITC as the protein ELP fusion becomes more concentrated (thus resulting in a lower transition temperature) and hence requires a lower concentration of salt to drive the ELP phase transition. Although salts such as NaCl have very poor ability to nonspecifically "salt-out" proteins, it is prudent to add the minimum amount of salt necessary to trigger the phase transition of the protein ELP fusion, as high salt concentrations increase the likelihood of coprecipitating contaminant proteins, especially for salts such as ammonium sulfate that are potent "salting-out" agents.



Source: http://doi:10.3791/51583

Figure 6: ELPs protein purification by ITC technique.

The thermal properties of ELPs provide a means of fusion protein purification that is an excellent alternative to traditional purification methods such as chromatography.

E. coli cells are collected from the culture media by centrifugation and lysed by sonication prior to purification by ITC (1). The ELPs transition is triggered with the addition of heat or salt (2) and aggregated insoluble ELPs is collected by centrifugation (3). The supernatant, containing soluble contaminants, is discarded while the pellet, containing insoluble ELPs coacervate, is retained (4) and resolubilized in cold buffer (5). The solution is again centrifuged (6) and the pellet, containing insoluble contaminants, is discarded, while the supernatant, containing the soluble ELPs, is retained (7). Steps 2 through 7 constitute one round of ITC, and Cycles of alternating hot and cold spins are repeated until desired purity is reached.

1. Add salt until the solution turns turbid. Centrifuge at 16,000 ×*g* for 10 min at room temperature.

↓

2. Remove the supernatant. Add ~750 µL of PBS (or chosen purification buffer) and resuspend pellet.

↓

3. Centrifuge samples at 16,000 ×*g* for 10 min at 4°C.

↓

4. Transfer the supernatant to clean tubes without disrupting the pellet. If possible, combine samples into a smaller number of tubes.

↓

5. Repeat steps 1-5 until sample is pure. Typically, 3-5 rounds of ITC produce a pure sample.

4.3.1 Evaluation and processing of purified protein ELP fusions

To verify the purity of the protein ELP fusion, the first step is to evaluate the purification product by SDS-PAGE analysis. Visualization of the protein band by SDS-PAGE verifies the presence or absence of contaminants as well as the molecular weight of the purified protein. SDS-PAGE should not, however, be relied upon to solely judge the molecular weight of the protein as ELPs can in some cases display anomalous migration in SDS-PAGE relative to protein standards (Meyer and Chilkoti., 2002). Other techniques such as mass spectrometry are more appropriate to precisely measure the mass of the protein.

1. Samples from each step of purification by ITC should be incubated with an appropriate loading dye for 2 min at 100°C.

↓

2. Load the sample onto a pre-cast SDS-PAGE gel and run until the dye reaches the end of the gel.

↓

3. Stain the gel with a 0.5 M $CuCl_2$ solution for 5 min or with a zinc solution according to the steps below:

    a. Incubate gel for 10-15 min in a 0.2 M imidazole, 0.1 % SDS (v/w) solution.

    b. Transfer the gel to a 0.3 M ZnSO4 solution and incubate for 30 sec (no longer than 45 sec).

    c. Rinse with water for 3 min.

To cleave the ELP tag if desired, the corresponding protease cleavage kit should be purchased. It is highly recommended that a biotinylated protease (if available) be used in this step as subsequent removal of the protease from the mixture can be accomplished with streptavidin agarose beads. After cleavage of the protein from the ELP fusion, pure protein can be recovered as follows:

1. Add the minimum amount of salt to transition the cleaved ELP.

↓

2. Centrifuge sample at 16,000×*g* for 10 min at room temperature. Save the supernatant.

↓

3. To remove the excess salt, the sample can be either dialyzed using the appropriate molecular weight cut-off dialysis membrane or filtered through a gel-filtration column.

↓

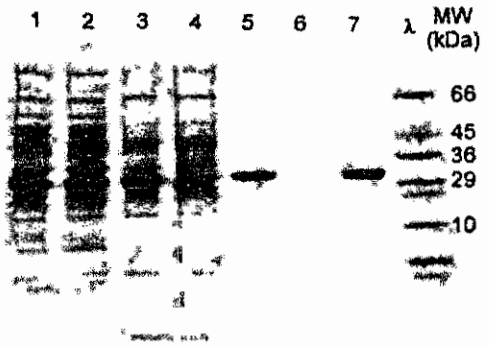
4. Repeat SDS-PAGE analysis to visualize the therapeutic protein and to ensure complete cleavage and purification from the protease and ELP tag.

For samples intended for *in vivo* applications, additional removal of endotoxin may be desired. Purified therapeutic protein can be passed through endotoxin removal columns

(such as Pierce's Detoxi-gel). The presence of endotoxin can then be quantified by a gel clot assay (such as Lonza's Pyrogent assay).

4.3.2 Gel Analysis

In examining the SDS-PAGE results, a single band should be observed for a single sub-unit protein that corresponds to the correct molecular weight of the protein ELP fusion, as seen in (Figure-7). Additional bands observed in that lane indicate contaminants that can be removed by additional rounds of ITC. If the lysed cell lane shows no overexpressed band at the molecular weight of the target protein ELP fusion, optimization of growth conditions (temperature, time, cell line and induction) is recommended. In addition, the choice of ELP guest residue composition or length should be reexamined.



Source: (Shamji et al., 2007).

Figure 7: SDS-PAGE analysis of protein ELP fusion purification by ITC.

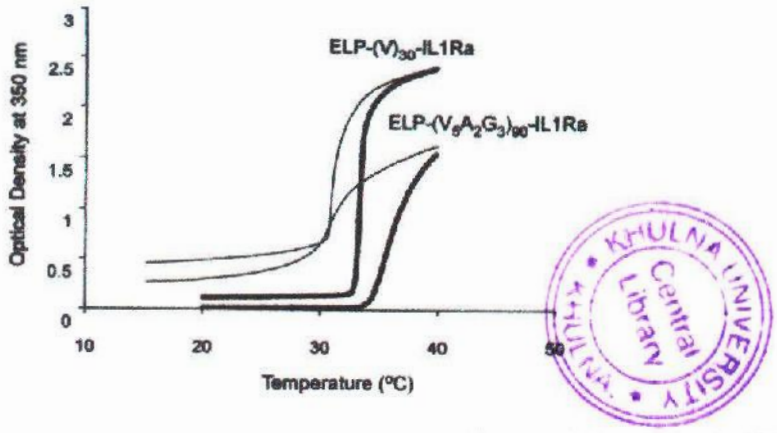
A therapeutic interleukin-1 receptor antagonist ELP fusion, with applications for osteoarthritis, was examined throughout purification with two rounds of ITC. Each lane indicates the following purification steps: (1) cell lysate; (2) soluble cell lysate; (3) soluble lysate after addition of PEI; (4) insoluble pellet after addition of PEI; (5) supernatant after first cold spin; (6) supernatant after first hot spin; (7) supernatant after second cold spin.

Note: The presence of the overexpressed protein ELP fusion band in early stages of purification and the singular band of pure protein ELP fusion achieved with only two rounds of ITC.

4.3.3 Physical characterization of protein ELP fusions

After cloning, expression, and purification of the protein ELP fusion, characterization of the thermal properties and higher order assembly are important to assess the applicability of the fusion to its intended application. Because the fusion of an ELP to a protein can alter the phase behavior, it is imperative that each fusion be evaluated with regard to its thermal behavior.

Evaluating the T_t of a protein ELP fusion provides the precise temperature at which ELP aggregation (or higher order self-assembly) will occur at a given concentration and buffer conditions. This thermal response can be measured by temperature-programmed turbidimetry where the light attenuation of the protein ELP fusion solution is monitored at 350 nm as the temperature is ramped, typically at a rate of 1 °C/min. The solubility of ELPs below their T_t leads to little light extinction. As the temperature is increased and approaches the T_t, the extinction of the solution will rapidly increase, indicating the coacervation of ELPs into micron size ELP-rich aggregates (Figure-8). The ELP T_t can be defined from this data as the temperature at which the first derivative of the absorbance reaches its maximum (otherwise defined as the inflection point of the absorbance curve). More subtle changes in the absorbance spectrum with respect to temperature can provide further information about nano-mesoscale assembly prior to the complete coacervation of the ELP. The assembly of ELP block copolymers into spherical micelles is one such example, where a region of increased absorbance occurs prior to aggregation in which the stable nano-scale ELP micelles contribute to the solution turbidity.



Source: (Shamji et al., 2007).

Figure 8: Thermal characterization of protein ELP fusion by temperature regulated turbidimetry.

The thermal properties of therapeutic fusions of interleukin-1 receptor antagonist (IL1Ra) with two unique ELP sequences were evaluated by temperature-programmed turbidimetry. Increasing the temperature (thick lines) leads to an increase in optical density at 350 nm and reveals transition temperatures of approximately 34°C and 35-40°C for an ELP fusion of 30 repeats with valine as the guest residue (ELP-(V)30-IL1Ra) and an ELP fusion of 90 repeats that contain valine, alanine, and glycine guest residues in a ratio of 5:2:3 (ELP-(V5A2G3)90-IL1Ra), respectively. Both ELP fusions demonstrate reversibility in their thermal transition as the optical density decreases as the temperature is reduced (thin lines).

Additional information about the thermal response of protein ELP fusions can be inferred from temperature-programmed light scattering. While temperature-regulated turbidimetry can provide the temperature at which an ELP or its fusion protein self-assembles or phase separates into micron size aggregates, it cannot provide information about the size and shape of the assemblies nor is it sensitive to low levels of adventitious aggregation of a protein ELP fusion. Dynamic and static light scattering are two powerful techniques that can supply this information, providing additional evaluation of the size, molecular weight, and topology of such assemblies (Schartl et al., 2007).

CHAPTER 5

CONCLUSION

CONCLUSION

Elastin like polypeptides is a stimuli-responsive biopolymers with tunable properties that biomaterial for applications in protein purification, drug delivery, tissue engineering, and more generally as biomolecular actuators. The environmental responsiveness of ELPs can be directly encoded with molecular precision into the sequence and architecture of the ELP and these designer biopolymers can be readily synthesized by recombinant DNA methods. This review highlights the various aspects of ELPs as polymeric carriers for drug delivery applications, protein purification, tissue engineering.

ELPs into versatile, customizable biomaterials for a variety of biomedical applications. Because they offer such exquisite control over their sequence, there are many opportunities to tune their mechanical, chemical, and biological properties. By tuning the thermally responsive behavior of ELPs or attaching bioactive groups, these polypeptides can be engineered for a variety of tissue engineering and drug delivery applications. More recently, groundbreaking research on ELP-based materials has introduced new architectures that can be harnessed to address the needs of the field.

Currently, ELPs works on the bacterial expression systems typically *E. coli* to produce the polypeptide. The appeal of this expression system is clear: bacteria rapidly produce high yields of the polypeptide. However, bacterial expression systems restrict the use of ELP fusion partners to peptides and proteins that do not require glycosylation and can properly fold in the bacterial cytoplasm.

Protein purification using ELP fusion tags and ITC is an easy and dynamic method for the purification of recombinant target proteins from cell lysate after expression. With these known applications, ITC is adjustable enough to ensure elaboration for a specific protein of interest using diverse ELP tags and solution conditions yet it remains standard enough to be useful for high throughput applications involving different proteins with varied physicochemical properties. In conclusion, ITC is a unique bio-separation technology that should have a significant impact on biotechnology and the biological sciences.

Therefore, ELPs in a wide range of biomedical and biotechnology applications requiring highly uniform, stimulus sensitive, and biocompatible materials. The mono-dispersity,

precisely defined composition and biodegradability of ELPs make them attractive for drug delivery. This article highlights the various aspects of ELPs as polymeric carriers for drug delivery applications especially cancer chemotherapy by exploiting their unique property, namely ITT, in addition to targeting tumors passively through their EPR effect. ELPs can be used for not only for conjugating anticancer agents but also other therapeutic peptides, like cell penetrating peptides, lactoferrin peptides, radionuclide conjugate, anti-proliferating peptides, penetrating for targeted delivery and multidrug resistance nanomedicine applications. At the present time focuses on ELPs for drug delivery applications to improve the therapeutic efficacy of pharmacological agents particularly anticancer drugs. It is possible that an increased understanding of drug delivery aspects and tailoring of ELPs to optimize their thermal control for pharmacokinetic clearance and also how the concentration of ELPs affect their ITT behavior may contribute newer dimensions to tumor targeting. Nanoparticle-based ELP-drug conjugates can also be investigated to enhance drug accumulation at tumor sites. These nanoparticle-based drug delivery systems may open up new frontiers for targeting to cancer by increasing tumor accumulation of anticancer drugs with reduced systemic toxicity. ELPs can also be possibly exploited for targeting in the case of metastasis. Current and future developments in polymeric-based drug delivery applications will no doubt open new frontiers in the next generation cancer chemotherapy. These various types of elastin like polypeptides application are poised to render more tangible the next era of healthcare and pharmaceutical science nanomedicine.

CHAPTER 6

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