Published in final edited form as:

J Control Release. 2014 September 28; 190: 304–313. doi:10.1016/j.jconrel.2014.06.016.

Controlled Release from Recombinant Polymers

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Abstract

Recombinant polymers provide a high degree of molecular definition for correlating structure with function in controlled release. The wide array of amino acids available as building blocks for these materials lend many advantages including biorecognition, biodegradability, potential biocompatibility, and control over mechanical properties among other attributes. Genetic engineering and DNA manipulation techniques enable the optimization of structure for precise control over spatial and temporal release. Unlike the majority of chemical synthetic strategies used, recombinant DNA technology has allowed for the production of monodisperse polymers with specifically defined sequences. Several classes of recombinant polymers have been used for controlled drug delivery. These include, but are not limited to, elastin-like, silk-like, and silk-elastinlike proteins, as well as emerging cationic polymers for gene delivery. In this article, progress and prospects of recombinant polymers used in controlled release will be reviewed.

Keywords

Recombinant Polymers; Controlled Release; Elastin-like Polypeptides (ELP); Silk-like Polypeptides (SLP); Silk-Elastin-like Protein Polymers (SELP); Recombinant Cationic Polymers (RCP)

Recombinant Polymers

Natural proteins have a predesigned function eliciting a specific cellular response. This automated behavior is resultant of their primary amino acid sequence that dictates folding patterns and architecture, which in turn commands function.[1] Understanding the central dogma of molecular biology has opened avenues in biomaterial design, particularly

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engineering recombinant protein polymers for new applications in biological systems. These recombinant polymers are synthesized by harnessing the protein synthesis machinery of cells through genetic engineering and DNA manipulation.[2–7] The sequences of recombinant polymers are often borrowed from naturally occurring proteins grounded on function. DNA sequences of diverse species may be combined or synthetic sequences using non-canonical amino acids like p-fluorophenylalanine or selenomethionine may be introduced, creating novel functions demanded by the chosen application.[8]

The early 1970s encompassed pivoting work by biochemists including Lobban, Kaiser, Berg, and Khorana who developed biochemical methods for inserting new genetic material in pre-existing DNA of a bacteriophage, paving the way for chemical syntheses of genes and recombinant DNA technology.[9–12]. One of the earliest works describing synthesis of a recombinant polymer in a biological system was by Doel and coworkers in 1980 where a defined DNA sequence was made via phosphotriester methodology and subsequently cloned into *E. coli* using plasmid vectors with controllable promoters.[4] *E. coli* expression yielded a simple polymer of phenylalanine and aspartic acid that is enzymatically degradable.[4] Synthetic methods of more complicated polymers composed of repetitive co-polypeptides began to be established in the late 1980s and early 1990s including work by Fournier, Tirrell, and Cappello.[2, 5, 8, 13–15] Both chemical synthesis and genetic strategies were explored. McGrath et al. described both techniques to create co-polypeptides $[-(\text{GlyAla})_3\text{-GlyProGlu-}]_n$, the first a purely chemical synthesis via classical solution phase methods, yielding a polydisperse product of average molecular weights less than 100,000 Da, followed by recombinant DNA methods where chemically synthesized oligomers were self-ligated resulting in multimers subsequently incorporated into expression plasmids with appropriate translation signals. *E. coli* expression of the plasmids yielded monodisperse product.[5] This work is a powerful example indicating key differences in polymer synthesis strategy.

Although, synthetic polymers have been designed in parallel with recombinant polymers for similar applications, there are several advantages to recombinant polymers over their chemically synthesized counterparts. First, the level of control by nature's cellular machinery to make macromolecules remains unsurpassed.[1] When recombinant polymers are made in a biological system, such as bacteria, the product is virtually monodisperse in both sequence and size. Each polymer strand is an exact copy generated from a DNA template, which was previously designed and transferred into the production cell line.[16] This monodispersity can be important in drug delivery applications to enable generation of distinct release profiles and determine structure-function relationships that may not be possible with a statistically defined polymer system. Chemically synthesized macromolecules cannot match this precision especially in more complicated and longer chain length polymers, which often generate statistical distributions of polymer lengths. Figure 1 depicts general recombinant protein engineering methodology, which begins with design of the amino acid sequence for the desired product. The DNA sequence is synthesized using solid phase techniques. Often, the final product consists of repeats of a single sequence; therefore a small oligomer is synthesized followed by a multimerization process and enzymatic ligation into a DNA plasmid vector. Multimerization can be considered a polycondensation event where designed sticky ends of the oligomers bind,

creating random length multimers. Once the multimers are ligated into the vector, a host cell, often a bacterium like *E. coli*, is transformed and grown as individual colonies for plasmid screening. The screening allows determination of multimer size and consequently isolation. Isolated plasmid is re-transformed into a host cell and large scale fermentation and purification of product ensues.[1, 8] Expression vectors are designed to have a switch, such that the protein is only expressed at high bacterial density. Switches may be chemically or temperature induced. Premature protein synthesis will hinder bacterial growth and product yield will be low. [1]

Second, recombinant polymers are often biodegradable as they are constructed of simple amino acid residues and degrade into small peptides or single amino acids.[17] Third, recombinant polymers can be systematically altered in order to generate libraries of potential candidates or investigate the exact role of individual components of the polymer system on properties important for controlled release such as mechanical integrity, bioactive agent release, biorecognition, degradation and elimination. The Urry group systematically approached the synthesis of peptide-based polymers to understand how different biophysical properties change with methodical change in polymer sequence.[18] They began by constructing a basic gene unit encoding (GVGVP)₁₀ and building concatemer genes with varying repeats of the monomer unit, creating macromolecules greater than 100,000 Da. This work, along with other work using sequential poly peptides synthesized chemically, generated a library of polymers relating structure to function whereby strategic changes in the nature and sequence of amino acid residues altered physicochemical properties of the polymers such as pH and temperature sensitivity and solubility.[17–19] Several classes of recombinant polymers are well-studied. Recent progress in these is reviewed below.

Elastin-like polypeptides

Elastin-like polypeptides (ELPs) in their most basic form are recombinant polymers comprised of repeats of the amino acid sequence valine-proline-glycine-X-glycine (VPGXG) where X represents any amino acid except proline as found in mammalian tropoelastin.[20–23] This repeating sequence forms a soluble β -helical structure at lower temperature, which reversibly collapses into an insoluble aggregate above a critical temperature termed the inverse transition temperature (T_i) that can be tuned using ionic strength in solution.[24, 25] The number of repeats and the guest residue X can be used to shift the transition temperature and impact other sensitivities such as electromagnetic fields, [26] ionic strength, [25] and pH.[27] The biocompatibility of ELP in its basic form using generic biological tests was extensively studied by Urry et al. [28] Their results indicated excellent biocompatibility, therefore establishing ELP as a parent biomaterial for development of a wide range of medical applications. Examined properties of ELPs in the formation of nanoconstructs and hydrogels are reviewed extensively.[23, 25, 29–37]

ELP based nanoconstructs

ELP nanoparticles were first fabricated with the synthesis of ELP di-block copolymers with distinct guest residues and block lengths leading to different transition temperatures (T_i). As the solution temperature is elevated above the lower T_i , this block collapses becoming

hydrophobic while the higher T_t block remains hydrophilic. The hydrophobic blocks associate to minimize hydration while the hydrophilic blocks form a hydrated corona making micelle structures.[38] This transition is also exploited in the purification of ELPs through reverse phase transition cycling during which ELPs can be selectively forced out of solution using varying amounts of salt and heat. This behavior is an advantage to the use of ELPs and leads to a much simplified purification compared to other recombinant proteins and chemical polymers. ELP micelles have been used in a myriad of applications to deliver a wide array of bioactive agents from chemotherapeutics such as doxorubicin[39] and geldanamycin[40] to growth factors such as bone morphogenetic protein 2 and 14[41].

More recently, research efforts have turned to using ELP carriers to increase the therapeutic efficacy of clinically relevant drugs by tuning the polymeric structure. One method being explored is the use of multiple-stimuli sensitive ELP-based particles designed to dissociate in the presence of the lower pH environment within solid tumors, which has shown promising results in increasing tumor targeting and penetration of drugs and imaging agents.[42] Along with targeted dissociation, multiple locations of drug attachment in the hydrophobic core and hydrophilic corona of ELP micelles have been achieved by the genetic engineering of a binding protein to the surface of ELP micelles. This attachment has shown the ability to increase sequestration and prolong the release of the hydrophobic drug rapamycin.[43] In a related study, this construct was shown to increase the effectiveness of rapamycin which suffers from dose limiting toxicity in the treatment of the inflammatory disease Sjögren's syndrome.[44] Further, the attachment of whole receptors or fragments to the corona of ELP micelles has been accomplished. [45] Previously, only short peptide fusions to ELPs had been attempted for targeting of micelles due to the potential for structural disruption of the particles.[46] The ELP micelle systems made by whole receptor fusion targeting strategy can tolerate fusions of over 100 amino acids and still retain function.[45] This has demonstrated another advantage to ELP based nanoconstructs in the ability to add large protein based targeting moieties into the innate structure of the nanocarrier without the need for additional chemical linking steps. An inverse targeting strategy has also been explored by administering chimeric ELP based peptide strands intravenously and heating an area of the body, in this case a tumor, above T_t . This lead to the assembly and anchoring of constructs in the tumor and vasculature termed thermal targeting, which has shown success with doxorubicin[47] and radioisotopes for brachytherapy.[48]

While strides were made in increasing the effectiveness of ELP micelle delivery, biodegradation and elimination are also important topics of study. It has now been shown that ELP micelles are subject to biodegradation in hepatocytes after uptake as expected; however, it appears the micelle structure is protective against some enzymes such as collagenase known to degrade ELPs in the soluble form.[49] Efforts were also made to form mathematical models to predict the behavior of ELPs in solution and micelle formation based on their length and guest residues to allow for a rational design of delivery systems tailored to the drug of choice.[50] This mathematical modeling approach was also attempted with ELP fusion proteins and has likewise shown success.[51]

Assembly of ELP micelles has traditionally relied on the T_t as described above, however recently other methods of assembly have also been shown. In one such instance T_t is

bypassed by the conjugation of hydrophobic moieties to soluble ELP strands which can trigger assembly into micelles.[52] This assembly is reminiscent of classical chemically based micelle assembly seen in other systems.[53] Assembly of ELPs has also been attempted in the intracellular space with some success and has been shown in cells transfected with plasmid producing ELPs.[54] This approach could lead to the development of intracellular structures or potential organelle targeting.

ELP polymer hybrids with other materials have been considered for the synthesis of more complex nanomaterials. In one case, cylindrical micelles were formed by radical polymerization of ELPs which may be advantageous in drug delivery due to the large number of potential sites for functionalization.[55] Combining the monodispersity and functional strengths of recombinant polymers with chemical polymerization can yield the design and development of a wide array of organized structures. Similar to ELP hybrids, the synthesis of multi-armed ELP constructs has been achieved that consists of an ELP domain fused to a fold-on domain that links three individual polymer strands.[56] These constructs have shown self-assembly into the expected spherical micelles at low salt concentrations, but forming elongated rods with an aspect ratio in excess of 10 in higher salt concentrations. [57]

The *in vivo* quantitative particle tracking of ELPs has been explored with positron emission tomography (PET) imaging in combination with ^{64}Cu conjugated polymers, as pharmacokinetics and tracking the fate of administered drug delivery systems has long been a mainstay of preclinical testing. It was shown the half-life and distribution of ELPs did not change based on whether they were assembled into micelle structures, but rather wholly depended on the molecular weight of the individual polymer strands. This result may show the micelles formed could be unstable in blood and may break into unimer strands, which would show the nearly identical pharmacokinetic parameters observed. [58]

Although earlier work had shown convincing biocompatibility of basic ELPs[28], newer constructs made from these polymers or modifications thereof need to be evaluated individually. Furthermore, architecture of the materials can affect distribution, degradation, and elimination. Final biological fate must be determined and quantified, particularly long-term responses by the immune system if nanoconstructs made from recombinant polymers are to be used clinically.

ELP based hydrogels

ELP hydrogels like their nanoconstruct counterparts are formed of the same basic structure of repeats of VPGXG, but often with other modalities designed into the polymer backbones to facilitate hydrogel formation. These hydrogels have been formed by several distinct mechanisms of varying effectiveness for biologic application. Cross linking strategies including chemical, [59] enzymatic, [60] photo-mediated, [61] γ -irradiation[62], and physical assembly [63, 64] have been used. More recently, additional methods of cross linking have been successfully tested utilizing cystine residues designed into the polymer backbone. These residues form disulfide bonds between strands, reducing the weight percent of polymer needed to form hydrogels although an oxidative environment is still required,

which is generated using chemical agents.[65] Rheological testing of these materials revealed lower storage moduli than expected that was attributed to a low number of elastically active disulfide cross links.[66] Additionally, ultrasonication was explored coupled with heating for hydrogel formation in GVGVP structured ELPs and subsequent concomitant release of model antibiotics and proteins showing apparent first order release kinetics.[67]

Depot based release of several drugs was attempted including the use of proteolytically degraded systems for the delivery of glucagon-like peptides to assist in the treatment of diabetes. These peptides were shown to be expressed as a fusion with ELPs and released by enzymatic cleavage[68] with activity in reducing blood glucose levels up to 5 days after administration showcasing the ability of ELP hydrogels to potentially use single step synthesis of depot and cargo.[69] Similarly, an injectable, gel forming ELP-curcumin conjugate for delivery of anti-inflammatory molecules to the sciatic nerve showed promise in limiting systemic dissemination of the drug and satisfactory release over 4 days.[70]

Degradation of enzymatically cross-linked ELP matrices was also recently tested by subjecting hydrogels to proteolytic enzymes from bacterial and mammalian origin. The digestion showed promising results liberating polymer strands from a cross-linked network and additionally showed an increase in release of a model protein, enhanced green fluorescent protein, upon degradation.[71] With regard to degradation, an area that remains to be fully explored includes final biological fate and geometry of digested gel fragments that leave the depot location. Moreover, toxicity of ELP conjugated with drug must be tested at off-target locations, as there is potential for strands of the network to release during degradation which continue to have a drug attached.

Silk and silk-like proteins

While ELPs have a predominantly conserved consensus sequence used in the majority of controlled release applications, silk and silk-like proteins (SLPs) have a number of commonly used sequences from several species of silk worms and spiders. Silk and SLPs have gained favor in the drug delivery and controlled release field for their mechanical strength, biocompatibility, and biodegradability. The basic properties of SLPs are reviewed extensively elsewhere.[72–76]

The most common of all silk variants is likely the *Bombyx mori* silkworm silk fibroin consisting of a light 25 kDa chain and a heavy 325 kDa chain. Silk fibroin has been widely used for controlled release of drugs and proteins from films, gels, implants, and nanoparticles [77–84]. While widely used, *B. mori* silk fibroin is generally extracted from natural sources and not synthesized by recombinant techniques. Attempts, however, have been made to express other silks such as spider dragline silk from *B. mori*. [85] Alternatively, recombinant silk from two species of spider *Nephila clavipes* and *Araneus diadematus* are widely mimicked for their favorable properties due to difficulty of isolating from natural sources.

SLP based materials

Recombinant spider silk based nanoparticles have been successfully fabricated and fused with other protein motifs to aid in cellular entry or targeting.[86–88] These methods have shown to repeatedly yield condensed protein particles capable of carrying drug and gene products. More recently, particles engineered to carry protein drugs have been fabricated in an aqueous process and showed colloidal stability over 24 hours at neutral pH and low salt, however pH 5 or increased salt resulted in significant particle agglomeration. Lysozyme as model cargo showed high loading of almost 30% w/w as well as penetration into the nanoparticle in addition to surface association. Release of loaded protein was maintained for 28 days and was dependent on the ionic strength of the solution.[89]

Screening for small molecular weight drug compatibility with SLP nanoparticles has also been performed. In this study particles composed of a 48 kDa protein consisting of 16 repeats of a sequence taken from *Araneus diadematus* silk protein ADF4 self-assembled into roughly 330nm particles upon titration with potassium phosphate. These particles showed greatest affinity for basic compounds with sufficient hydrophobic character to interact with the formed particle structure. Acidic molecules were much less tolerated. Model compounds showed sustained release for as long as 30 days, however acidic conditions resulted in increased burst release on day one, demonstrating potential for delivery to the acidic tumor microenvironment.[90] Composites of the ADF4 based recombinant protein and classic controlled release materials such as poly(caprolactone) (PCL) and polyurethane (PU) were also explored and showed a drastic reduction in the Young's modulus and tensile strength of the material compared to the SLP alone. The study also showed changes in release of model drugs based on the addition of PCL and PU over 30 days which indicates a potential for tuning the release of compounds to desired rates for a given drug.[91] The mechanical properties of potential SLP based particles have been studied using recombinant ADF4 from *Araneus diadematus*. A remarkable mechanical stability was observed with dry particles, but was drastically reduced upon hydration. It was also found that the modulus of particles could be tuned by changing the length of the expressed recombinant protein and with the addition of cross linking of polymer strands. Most interestingly, changes in mechanical and swelling properties from a dry to hydrated state were completely reversible showing feasibility of long term storage of a drug delivery or cosmetic product.[92]

SLP hybrids with cationic blocks have shown utility in tumor targeted delivery of plasmid DNA. SLP sequences based on the MaSp1 protein of dragline silk from *Nephila clavipes* together with a 15-lysine residue block and tumor homing peptide have shown condensation with plasmid DNA into 100nm particles. These complexes were further optimized for size and surface charge. The resultant particles showed low cytotoxicity, but also much lower transfection than a lipofectamine control demonstrating proof of concept, but in need of further optimization.[93]

SLPs have been compared to expressed proteins from *Nephila clavipes* by computer modeling. This work showed successful prediction of several key parameters of the fiber form and films created from two different SLP analogues such as water contact angle, and used predicted degree of beta sheet formation for an indicator of material strength.[94]

Importantly, time and size scales used for modeling of proteins were not matched to real world observations of silk proteins but still provided predictions of macroscopic behavior. These findings show the possibility of modeling SLP sequences to aid in rational material design in addition to the potential ability to predict structural consequences to the addition of *de novo* sequences in SLPs. Results from this study could be the first steps to complete *in silico* design of protein sequences.

The utility of wrapping silk from *Argiope trifasciata* in the preparation of nanoparticles has been explored. The aciniform protein AcSp1 investigated showed self-assembly into nanoparticles of 50nm and 220nm without the appreciable presence of monomers left in solution. Particles showed good stability in a range of buffered solutions and reportedly had thermal stability past 70° C. Loading of rhodamine B as a model drug was also investigated. [95]

Silk-elastinlike protein polymers

Silk-elastinlike protein polymers (SELPs) are block copolymers consisting of amino acid repeats derived from *B. mori* silk (GAGAGS)[96] and mammalian elastin (GVGVP).[97] SELPs were originally developed in the late 1980's to early 1990's for a variety of indications[98] and typically exhibit a sol-to-gel transition when more than two silk blocks are present in the polymer with increasing transition temperature tuned based on increasing block lengths.[99, 100] From the original synthesis of SELPs, many variants have been developed through the addition of guest residues in elastin blocks and altering the length and ratios of the silk and elastin blocks which can be used to alter resultant polymer and hydrogel characteristics.[101–106] Detailed synthesis and characterization, and biological evaluation of SELPs have been reviewed extensively.[33, 99, 100, 107–109]

The SELPs initially used for controlled release applications were developed in the late 1990's through the 2000's, namely SELP47K, [110] SELP415K, [101] and SELP815K[102] (for amino acid sequences see Figure 2). These constructs, composed of 4:8, 4:16, and 8:16 ratios of silk blocks to elastin blocks per monomer repeat respectively with a single lysine substituted elastin block (GKGVP) per monomer, were biosynthesized to examine the effects of silk to elastin block ratios, lengths and sequences on the physiochemical properties of resultant hydrogels. Tight control over the primary sequence of these polymers afforded by recombinant DNA technology was vitally important to effectively determine the contribution of each length and ratio of silk and elastin to final polymer properties. It was found that altering the composition ratios and sequence had significant effects on soluble fraction, [102, 104, 111], swelling ratio, [101, 102, 111, 112] and mechanical strength[101, 102, 113] of resulting hydrogels. These findings lead to the ability to tune release of bioactive agents based on size and surface characteristics.

SELPs have been examined for controlled release of markers and bioactive agents from 180 Da in the case of theophylline[114] to approximately 110nm particles in the case of model polystyrene beads.[115] Low molecular weight substrates under 13kDa have often shown fast release from SELP47K at 12 wt% in under 4 hours.[114] By increasing the concentration of polymers forming the hydrogels to 20 wt%, 85% release of a 500 kDa

model dextran was achieved over 10 days and release of a 40kDa dextran was achieved over 3 days from SELP47K.[110] Surface charge also been shown to affect release rates from SELP hydrogels with positively charged molecules releasing more slowly than negatively charged where positive, negative, and amphoteric analogues of rhodamine were used.[116] Gene delivery from SELPs has also been explored firstly by examining the characteristics of plasmid DNA release which showed highly tunable characteristics releasing from 2% to 80% plasmid load over 28 days while DNA concentration showed little effect on cumulative release rate.[100, 117] Despite the highly controllable release of naked plasmid DNA, this route of gene transfer often lacks the efficacy required for most applications and as a result viral release from SELP hydrogels has been explored. Delivery of adenoviruses from SELP47K over 28 days showed polymer concentration to be the key parameter determining release rate, [118] however, release also showed dependence on polymer construct as SELP415K released faster than equivalent concentrations of SELP 47K due to the higher elastin to silk ratio leading to a less mechanically robust hydrogel likely having a larger pore size in the polymer network.[119] Consequently, these two parameters, structure and concentration of SELPs, can be easily manipulated to tailor release to desired levels. In addition, degradation of the hydrogel matrix can contribute to observed release rates with elastase based degradation showing up to 40% increase in release of plasmid DNA from SELP415K hydrogels.[112] SELP systems were also shown to increase the safety of adenoviral administration *in vivo* while increasing the efficacy of treatment and restricting viral load more tightly to tumor tissue than free viral injections.[105, 106] Additionally, biocompatibility of SELPs has been evaluated in subcutaneous and intradermal injections of SELP47K in guinea pigs. This study showed no local signs of reaction that could be considered clinically significant or harmful at 3, 7, or 28 days when assayed histologically using hematoxylin/eosin and Masson's trichrome staining. Moreover, cellular penetration into the periphery of the gel depots was observed.[110]

Among the SELP analogs studied, SELP815K, with 8 silk block and 15 elastin block repeats with an additional lysine substituted elastin unit (GKGVP) (Figure 2), exhibited the most favorable transfection profile for adenoviral delivery to xenograft head and neck squamous cell carcinoma solid tumors (Figure 3).[104, 105] Based on these findings, optimizing SELP815K for delivery was performed. In the process, the utility of SELP815K was compared to poloxamer 407, an off the shelf, chemically synthesized, poly(oxyethylene) poly(propylene oxide) block copolymer examined for matrix mediated viral gene delivery to solid tumors. [120, 121] In these studies delivery from SELP815K, using identical intratumoral injection conditions, demonstrated greater tumor size reduction, longer time until tumor size rebound after treatment, and statistically significant improvement in survival. These studies also showed progressive encapsulation of the bulk SELP material instead of the expected complete degradation when injected subcutaneously in non-tumor bearing mice.

Studies that showed the presence of gel material encapsulated and still present in animals beyond 12 weeks indicated an increase in the degradation rate of the bulk material was necessary.[120] This lead to the synthesis and characterization of SELPs with matrix metalloproteinase (MMP) degradation sequences designed into the polymer backbone due to

the well-known role of MMPs in tumor lifecycle and invasion.[122–126] MMP responsive SELPs were designed and showed an increase in degradation as evidenced by protein loss, loss of mechanical properties, and increased cargo release in the presence of MMPs (Figure 4).[115] Current work focuses on design and development of new generations of MMP responsive SELPs with degradable sequences located in strategic regions of the polymer backbone to correlate polymer structure with degradation rates and subsequently release and efficacy.

The potential mechanism for SELP gelation was examined using polymers with silk to elastin ratios of 1:8, 2:8, and 4:8 with distinct silk and elastin block regions afforded by tight sequence control and a tyrosine substituted elastin unit (GYGVP) in each of the elastin blocks. The constructs termed SE8Y, S2E8Y, and S4E8Y respectively with similar molecular weights were investigated.[127] Scanning electron microscopy and atomic force microscopy showed micelle like structures, which are hypothesized to precede gel formation. It was also shown that as the silk component of the polymer is increased, the reversibility of association decreases as assayed by circular dichroism, turbidity, and dynamic light scattering measurements.[127] These results demonstrate the proposed two-step mechanism for SELP hydrogel formation and also confirm the observation of increasing silk character in SELPs results in less reversibility once strands become associated. This also demonstrated the importance of tight control over polymer sequence as block discontinuity could drastically influence the proposed gelation mechanism.

Furthermore, a SELP polymer with a 2:8 silk to elastin ratio with additional lysine substituted elastin unit was subsequently modified with retinal using Schiff base chemistry. The resultant retinal-SELP construct showed shifts in optical properties as measured by optical polarization spectral analysis, which was hypothesized to be due to the innate properties of retinal shifting from a relaxed trans state to a more stressed cis state when excited with polarized light. This change in structure could possibly be used to impart photosensitive properties to SELP hydrogels for the purpose of drug delivery and controlled release.[128]

Due to the highly controllable nature of recombinant polymers with the ability to tightly control the sequence of expressed systems, SELPs have shown the ability to precisely control the release of cargo based on concentration, silk to elastin ratio and sequence, and degradation properties. Experiments assaying the safety of these polymers up to 12 weeks have shown a lack of significant toxicity or immune interaction as evidenced in subcutaneous injection in mice, and subcutaneous and intradermal injection in guinea pigs, but more complete analysis needs to be done in multiple relevant species to show the long term safety for clinical application.

Recombinant cationic polymers

Cationic polymers have been explored to transfer genes to mammalian cells, often using synthetic polymers such as poly lysine or polyethyleneimine (PEI). The development of recombinant cationic polymers (RCPs) has shown promise in correlating structure with function in non-viral gene delivery.[129] Bi-functional RCPs were first developed in the late

90's and early 2000's with a system composed of a cationic lysine oligomer to condense plasmid DNA coupled to a β -galactosidase derived protein with RGD functionalities for targeting cell surface integrins.[130] While providing proof of concept, this system lacked an efficient endosomal escape mechanism, and therefore sufficient efficacy. Bi-functional RCPs were also synthesized on a cationic oligomer block fused to an ELP, but in the absence of an endosomal escape mechanism the system lead to poor efficiency.[131] Tri-functional biopolymers were then synthesized in an attempt to achieve efficient gene transfer from RCPs. These polymers consisted of lysine-histidine (K-H) residues with a fibroblast growth factor 2 (FGF2) fusion. As in other constructs, the lysine residues condensed DNA while the histidine residues were hypothesized to promote endosomal escape through the proton sponge effect. In these studies it was found that a random sequence of K-H did not perform as well as distinct blocks of K and H. It was also determined that the incorporated H residues were not enough to promote efficient escape. [132] Multifunctional RCPs were next synthesized with four parts including arginine-histidine R-H domains for nucleic acid condensation and endosomal escape, a targeting peptide for delivery, a fusogenic peptide for endosomal escape, and a nuclear localization signal for intracellular trafficking. This 4 domain RCP showed increased gene transfer efficiency as compared to previous constructs.[133] This ability to stack new functional segments on identical clones of previously synthesized constructs is unique to recombinant polymers and represents one of the chief advantages of this polymer type. Since each previous construct can be replicated via its DNA sequence with near monodispersity, the consequences of insertion of additional segments can be studied without worry of batch to batch or molecule to molecule variability. RCPs show this advantage leading to stepwise improvement in material properties over time. A review on the development of RCPs can be found elsewhere.[129]

The multifunctional RCPs have been tested *in vivo* with a gene for thymidine kinase used in conjunction with the prodrug ganciclovir for suicide gene therapy. Delivery of the construct to xenograft SKOV-3 tumors with resultant tumor knockdown for 3 weeks was demonstrated.[134] This *in vivo* test of the RCPs is an important step past cell studies to show the feasibility of the vectors in more complex systems. Multifunctional RCPs have been improved with the incorporation of novel domains such as a DNA condensing block based on repeats derived from histone H1. This construct showed good transfection efficiency, greater than a PEI control in some cases with low associated cytotoxicity *in vitro*. [135] A domain derived from histone 2A was also explored for more efficient release of nucleic acids from carriers that had previously been achieved with other cationic condensing recombinant sequences.[136] This study showed low immunogenicity and toxicity of the new histone based polymer sections and higher release efficiency of nucleic acids, which leads to greater efficacy of treatment *in vitro* and *in vivo*.

Work has continued on the endosomal escape mechanism of RCPs with additional fusogenic peptides being tested.[137] These fusogenic peptides commonly used for endosomal escape were also compared head to head in order to determine the potential benefit of each sequence in the context of gene delivery from RCPs. Of the five sequences tested, a peptide known as GALA consisting of a glutamic acid enriched sequence derived from influenza

virus hemagglutinin showed the greatest effect with the least cytotoxicity. Additionally, these studies showed the versatility of recombinant polymers in the evaluation of potential candidates for controlled release. Through recombinant DNA technology, virtually identical protein polymer constructs were designed and expressed differing in only one key component, which allowed an effective head to head comparison and selection of the best candidate.[138] Delivery of siRNA has recently been shown to be effective from RCPs as well. A multifunctional RCP using similar domains to those shown in the past without a nuclear localization signal showed efficient delivery of siRNA to the cytoplasm of cells *in vitro* and adequate resultant gene silencing.[139]

Other Recombinant Protein Polymers

In addition to the body of work briefly reviewed above, there are other avenues that are being pursued with recombinant based polymers for controlled release. Collagen as a recombinant peptide sequence has been explored, although to a lesser extent than silk since collagen can be found from natural sources abundantly including bovine hides and can be easily extracted from these sources. A problem with polydispersity arises if the desired collagen is to be a specific uniform length[140], fused with other proteins, [141] or a pure form of a specific collagen.[142] In these cases, recombinantly produced collagen and collagen-like proteins can be superior to their natural counterparts due to the ease of modification of the recombinant system and reduced batch to batch variability.

Challenges, Future Directions

Controlled release in a biological environment typically entails long-term residence of the delivery system. Long-term biocompatibility of recombinant polymers remains an area requiring further exploration. SELPs delivering viruses, for example, have been studied *in vivo* on a scale of weeks, where histological analysis of the surrounding tissue revealed a low inflammatory response, but quantitative interaction with the immune system has not been investigated. Establishment of quantitative immunogenicity will become more imperative as additional degradable systems are designed for controlled release, particularly systems using non-canonical amino acids and sequences from different species. For degradable systems, fine tuning the rate of degradation remains a challenge. Designing stimuli sensitive polymers requires detailed understanding of the environment where the recombinant polymer system will reside. In a diseased environment, a tumor for example, the local enzymes will be different or expressed at altered concentrations as compared to normal milieu. Biorecognizable motifs incorporated into the recombinant polymer identified by local enzymes in the diseased milieu may provide an effective strategy for rate control. However, structure and function of the polymer may be compromised. Furthermore, the tertiary structure of the designed recombinant polymer and targeting local enzyme must be well characterized to determine compatibility for interaction and desired outcome. Finally, high production costs remain a challenge in this field, particularly scaling up production and purification of recombinant polymers for commercial use.

Despite these challenges the true usefulness of recombinant DNA technology is only starting to be realized with the rise of inexpensive computing power which may lead to the eventual

ability to correlate protein sequence to structure and function. Currently modeling recombinant proteins after sequences derived from nature has been widely shown to be a formula for successful assignment of functionality. As this modeling becomes more powerful and available the production of designer proteins with specific engineered functions from novel amino acid sequences will become possible. In addition development of combinatorial approaches for the synthesis of a myriad of polymer structures at the same time and their subsequent high throughput evaluation can substantially advance this field. By nature many recombinant polymers can be immunogenic. Fusion with peptides or alteration of sequences such that immunogenicity is reduced is of paramount importance for successful translation. The high degree of control over incorporation of biomimetic structures within a well-defined structure promises a systematic structure function relationship in biomaterial design for controlled release.

Concluding Remarks

The favorable characteristics of biodegradability, near perfect monodispersity and the ability to correlate structure with function have led to the increased use of recombinant polymers in controlled release applications. The ability to elucidate the contribution of each polymer segment to observed macromolecular properties has allowed for efficient optimization. This ability also distinguishes recombinant polymers from their chemically derived counterparts whose polydisperse nature does not afford such benefits. Recombinant polymers will continue to evolve as a viable platform for controlled release as our knowledge of protein sequence from natural sources increases and the ability to model the secondary and tertiary structure of *de novo* sequences becomes more accurate.

Acknowledgments

Financial Support was provided by the NIH grant (R01-CA107621), a PhRMA Foundation pre-doctoral fellowship (RP), and a NIH pre-doctoral fellowship (1F30CA176922) (AP).

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Figure 1.
Generalized schematic of recombinant polymer synthesis

<i>Silk block</i>	<i>Elastin block</i>	<i>Lysine Elastin Block</i>	<i>MMP Responsive Sequence</i>
GAGAGS	GVGV	GKGV	GPQGIFGQ
SELP47K:			
GAGSGAGS [(GVGV) ₄ GKGV(GVGVP) ₃ (GAGAGS) ₄] ₁₂ (GVGV) ₄ GKGV(GVGVP) ₂ (GAGAGS) ₂ GAGA			
SELP415K:			
GAGSGAGAGS [(GVGV) ₄ (GKGV)(GVGV) ₁₁ (GAGAGS) ₄] ₇ (GVGV) ₄ (GKGV)(GVGV) ₁₁ (GAGAGS) ₂ GAGA			
SELP815K:			
[GAGS(GAGAGS)₂(GVGV)₄GKGV (GVGV)₁₁(GAGAGS)₅GAGA] ₆			
SELP815K-MMPRS:			
[GAGS(GAGAGS)₂(GVGV)₃GVGGPQGIFGQPGKGV(GVGVP)₁₁(GAGAGS)₅GA] ₆			

Figure 2.

Amino acid sequence of selected silk-elastinlike protein polymers. Note: Single letter amino acid code is used, head and tail sequences omitted for clarity.

Figure 3.
 β -galactosidase expression in xenograft tumors injected with adenovirus released from SELP47K (47K), SELP415K (415K), and SELP815K (815K) assayed at weeks 1, 2, and 3. Reproduced with permission from [104].

Protein Loss from Degradation

Particle Release with Degradation

Figure 4.

Protein loss (top) and particle release (bottom) as a result of digestion of SELP815K (left) and SELP815K-MMPRS (right) gels by matrix metalloproteinase enzymes. Reproduced with permission from [115]

Table 1

Common amino acid sequences and main structural components of recombinant polymers discussed in this review.

Recombinant Polymer	Consensus Sequence	Representative Structural Motif	Ref
ELP	(VPGXG) _n (X is any amino acid other than proline)	Temperature-induced phase separation behavior, resulting in aggregation and formation of an insoluble coacervate. Cysteine can be added for conjugation, or lysine residues for crosslinking.	[21,22]
Di-block	(GVGVVP) _m (GXGVP) _n (X is any amino acid other than proline)	Self-assemble into spherical micelles for given lengths and ratios of hydrophobic and hydrophilic blocks.	[21,22]
Tri-block	[(VPAVG)-(IPAVG) ₄] _m [(VPGXG) _n][(VPAVG)-(IPAVG) ₄] _p (Middle hydrophilic sequence less conserved)	Hydrophilic block capped on both ends by hydrophobic blocks will lead to spherical or cylindrical worm-like micelles	[21,23,63]
SLP			
Spider Silk	(G) _m (A) _n , GPGXX (X is most likely Glutamine, but can vary)	Repetitive alanine and glycine create high tensile strength crystalline structures. The pentapeptide contributes to formation of β-spirals.	[76]
Silk worm	(GAGAGS) _m , (GAGAGY) _n , (GAGAGA) _p or (GAGYGA) _q	The hexapeptides form crystalline β-sheet structures due to the dominance of hydrophobic Domains	[76]
SELP	[(GVGVVP) _m GKGVP(GVGVVP) _n (GAGAGS) _p] _q	Physical networks form as silk-like units collapse into β-sheets, and elastin-like units collapse into helical structures contributing to porosity	[33]

ELP: Elastin-like Polypeptides, SLP: Silk-like Proteins, SELP: Silk-Elastinlike Protein Polymers