REVIEW

TITLE: Nanotechnological approach to the therapeutic delivery using elastin-like recombinamers

José Carlos Rodríguez-Cabello*, María Jesús Piña, Arturo Ibáñez-Fonseca, Alicia Fernández-Colino, and Francisco Javier Arias.

BIOFORGE (Group for Advanced Materials and nanobiotechnology), CIBER-BBN, University of Valladolid, Valladolid, Spain.

ABSTRACT

This review discusses the use of elastin-like polymers and their recombinant version, elastin-like recombinamers, in drug-delivery systems. These macromolecules exhibit a number of interesting properties that are rarely found together in any other family of materials, especially extremely high biocompatibility, high bioactivity and functionality, complex yet fully controlled composition and stimuli responsiveness.

Appropriate design of these molecules opens up a broad range of different possibilities for their use in new therapeutic platforms. The first of these described herein is the use of ELRs in single-molecule devices as therapeutic entities on their own. Subsequently, we describe how the self-assembly properties of these materials can be exploited to create nanocarriers and, eventually,

microcarriers that are able to temporally and spatially control and direct the release of their drug load. Intracellular drug-delivery devices and nanocarriers for treating cancer are among the uses described in that section. Finally, the use of ELRs as base materials for implantable drug depots, in the form of hydrogels, is discussed.

1. INTRODUCTION

Besides the obvious essential role of pharmacology in the development of therapeutic agents, materials science also plays a pivotal role in this task. Thus, pharmacology aims to find new therapeutic molecules, whereas the field of biomaterials provides a platform for the development of a wide range of formats as delivery vehicles which, compared to the administration of free drugs, can provide advantages such as improved stability, solubility and *in vivo* pharmacokinetics.[1, 2]

Protein-based biopolymers are excellent candidates for the design of vehicles for drug-delivery purposes since their properties are determined exclusively by the physicochemical properties of their component monomers and their sequence.[3] The 20 chemically distinct natural amino acids can be combined in an almost infinite manner, thus providing high versatility and allowing a rational and precise design of protein-based materials to fulfill specific therapeutic requirements.

This review is focused on describing the engineering of elastin-like polymers (ELPs) for therapeutic applications.[4-6] As their name implies, ELPs are polypeptides whose sequence is bio-inspired by that found in natural elastin. Thus, the most broadly used motif displays the sequence $(VPGXG)_n$, where the guest residue X is any amino acid except proline and *n* symbolizes the number of pentapeptide repeats in the ELP. In order to ensure strict control over the sequence, chain complexity and monodispersity, recombinant DNA technology has been

implemented to bioproduce this class of materials.[7] Indeed, a new term, namely elastin-like recombinamers (ELRs), has been coined to refer to those ELPs produced using genetic engineering techniques.[8-10]

Besides their specific properties with regard to a particular design, the mimicry between elastin and ELRs can also be seen in their similarity as regards other properties, such as their biocompatibility, mechanical properties and stimuli-responsive behavior. This behavior is the result of a phase transition that the ELR experiences in response to an environmental stimulus such as pH, salt or temperature, amongst others.[11] The most common variable exploited to achieve such a transition is the temperature, which has led to the definition of the parameter "Tt" as the specific temperature above which the polymer chain remains hydrated, and below which the ELR chain folds hydrophobically and assembles to form a separate phase. In this folded state, the polymer chains adopt a dynamic, regular, nonrandom structure known as a β-spiral.[12, 13] Indeed, the fact that the polymer transforms thermal energy into mechanical work has led these polymers to be described as molecular machines [11] and the overall process as an inverse temperature transition (ITT).[14, 15]

Such stimuli-responsive behavior is especially relevant when engineering smart devices that are able to sense their environment and response to changes in it,[16] since this closely mimics the behavior of the human body, which is able to detect surrounding stimuli, also known as "inputs", and elaborate a response, or "output", in order to exert tight control over the resulting physicochemical process and correct potential imbalances **(Figure 1)**.

All the features listed above have provided a significant impetus to the development of various elastin-based platforms as carrier systems for the delivery of a variety of payloads (e.g. drugs, proteins, peptides, nucleic acids) in a variety of formats (hydrogels or depots, nanoparticles, monomers) over the past few years. As such, this review focuses on describing the most significant approaches in this field.

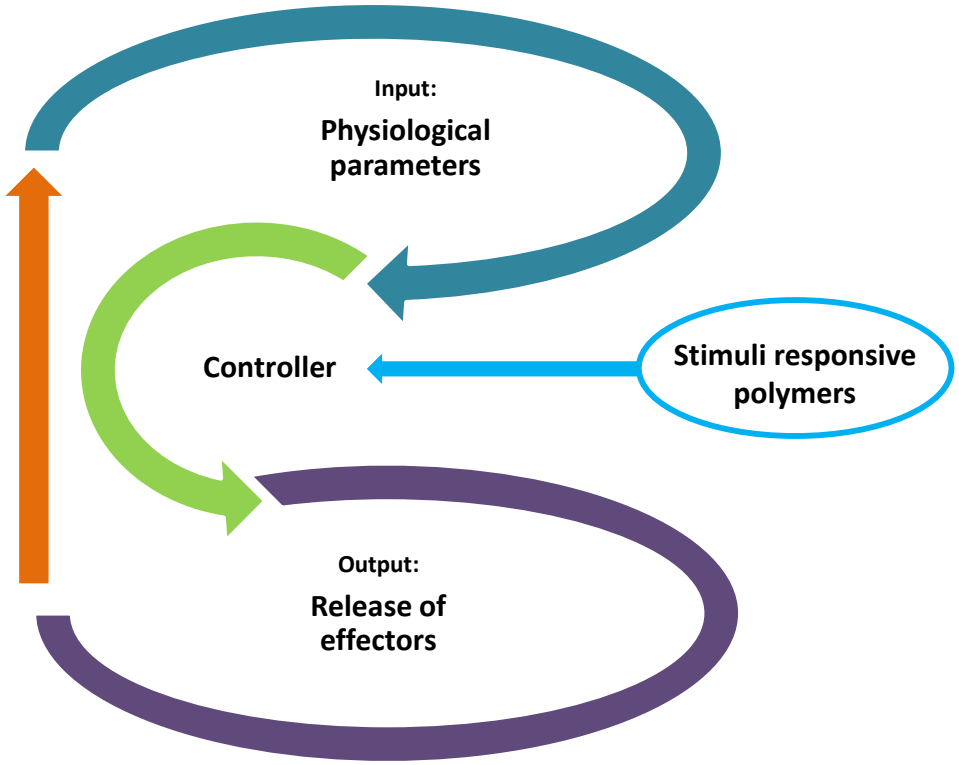

Figure 1. Scheme showing the physiological homeostatic feedback exerted by the human body. Such a loop arrangement ensures strict control over the physiological parameters. Recent trends in drug delivery try to emulate this loop arrangement by exploiting the environmental sensitivity exhibited by stimuli-responsive polymers.

2. Monomeric ELRs

Monomeric elastin-like recombinamers can be described as single-protein molecules adapted to have a function in therapeutics. They can be fused to therapeutic proteins in a recombinant manner for use as purification tags, as soluble delivery systems or as pharmacokinetic enhancers themselves, either by chemical conjugation or by fusion-protein recombinant expression of the drug.

2.1. ELRs as purification tags

One of the major issues in the downstream processes for obtaining recombinant proteins for therapeutic purposes is their high cost due to the need for chromatographic and other costly and

time-consuming product-purification techniques.[17] To address this problem, ELRs can be used as purification tags by taking advantage of a simple, costless and high-yield purification approach that relies on the ITT described above, in which the recombinamer can be obtained pure after several heating and cooling cycles following by centrifugation in a process called inverse temperature cycling (ITC). In addition, the ELR-based polypeptide can protect the protein/peptide from proteolytic degradation and from unfolding during the purification process. Further separation requires excision of the ELR tag and isolation of the target therapeutic protein, which is usually achieved by an additional heating and centrifugation step in which the ELR precipitates and the drug remains soluble in the supernatant. This method is useful only when the target protein is thermally stable at mild temperatures (37-42°C).

Under this premise, several approaches, which mainly differ as regards the tag cleavage strategy, have been developed to enhance the downstream process of recombinant drugs **(Table 1)**. One of these methods involves the inclusion of a self-processing module (SPM) from *Neisseria meningitides* FrpC protein, which undergoes autocatalytic cleavage between the Asp^{414} and Pro^{415} peptide bond at physiological concentrations of calcium ions.[18] This SPM has been successfully inserted into ELR constructs fused to green fluorescent protein (GFP), to the Fc portion of porcine IgG (pFc) and to human β defensin 3 (HBD3). After expression in *Escherichia coli*, the resulting ELR fusion polypeptides were purified by ITC and the product was attained by cleavage in a Ca^{2+} solution. Production yields ranged from 1.1 to 36 mg/L and purities from 90% to 100% of the final cleaved products.[19]

A similar methodology involves the addition of a self-cleaving intein sequence involved in protein splicing.[20] This sequence auto-cleaves when dissolved in a saline pH 6.0 buffer at room temperature and was first evaluated for bioseparations by Wood et al.[21] It was subsequently inserted into an ELR by this same group and the ELR-intein gene was fused to those coding for different proteins, such as β-galactosidase, catalase, GFP or maltose binding protein (MBP),

amongst others, to finally obtain highly pure products with specific activity when expressed in *E. coli* after ITC and cleavage reaction.[22] This same strategy was used in the purification of ELR-intein-tagged proteins in high cell density *E. coli* fed-batch fermentations[23] and for the production of recombinant *E. coli* RNA polymerase.[24]

Diverse antimicrobial peptides (AMPs) have been specifically expressed fused to an ELR tag for easier purification. Thus, halocidin18 (Hal18), which possesses strong antimicrobial activity against *E. coli* and *Micrococcus luteus*, was cloned into an ELR amino acid sequence and expressed in *E. coli*, obtaining 69 mg/L of the fusion product. Further excision of Hal18 was achieved by the addition of hydroxylamine cleavage reaction buffer and incubation at 55°C for 24 h, finally giving a recovery of approximately 47% of the product (1.7 mg/L) and a moderate purity of 60%.[25] Alternative AMPs, namely moricin CM4 and human β-defensin 4 (HβD4), were fused to an ELR tag, expressed in *E. coli* and cleaved due to the inclusion of an intein self-cleaving sequence, although the final yield of the target protein was not high (0.6 mg/L for CM4, 5.5 mg/L in the case of the lone recombinant protein,[26] and 1.8 mg/L for HβD4, compared to 5 mg/L of the recombinant AMP[27]).[28] Furthermore, the antimicrobial peptide cecropin AD (CAD) was fused to a cationic ELR (selected from previous studies[29]) for ITC purification, and a high purity CAD was obtained after expression in *E. coli* with a 12 mg/L yield (compared to 11.2 mg/L of the recombinant CAD[30]). In this case, separation of the ELR from the target AMP was performed by proteolysis with enterokinase.[31]

Table 1. Production yields for different proteins after excision from the ELR tag using diverse approaches.

excision approach	target protein	yield of the final product (mg/L)[a]	references
FrpC	GFP pFc HBD3[b]	1.1 to 36	Liu et al. ([19])

Intein	β-Gal	122.3	Banki et al. ([22])
	Catalase	79.8	
	GFP	110.2	
	MBP	46.4	
	OPH-S5	83	Fong et al. ([23])
	CM4[b]	0.6	Shen et al ([28])
	HβD4[b]	1.8	
Hydroxylamine cleavage	Hal18[b]	1.7	Hu et al. ([25])
Enterokinase proteolysis	CAD[b]	12	Yang et al. ([31])

[a]mg of final product per L of culture medium.
[b]Antimicrobial peptides (AMPs).

2.2. Therapeutic monomeric ELRs

Therapeutic elastin-like recombinamers are a promising tool for the design of recombinant drugs as this recombinant system adds new features to the drug itself in terms of specific delivery and improved pharmacokinetics. There are two different approaches for this purpose: chemical conjugation of the drug to the ELR via reactive groups, which allows the use of protein and non-protein drugs, or engineering of fusion recombinamers (ELR-drugs) in the case of protein-based therapeutics **(Figure 2)**.

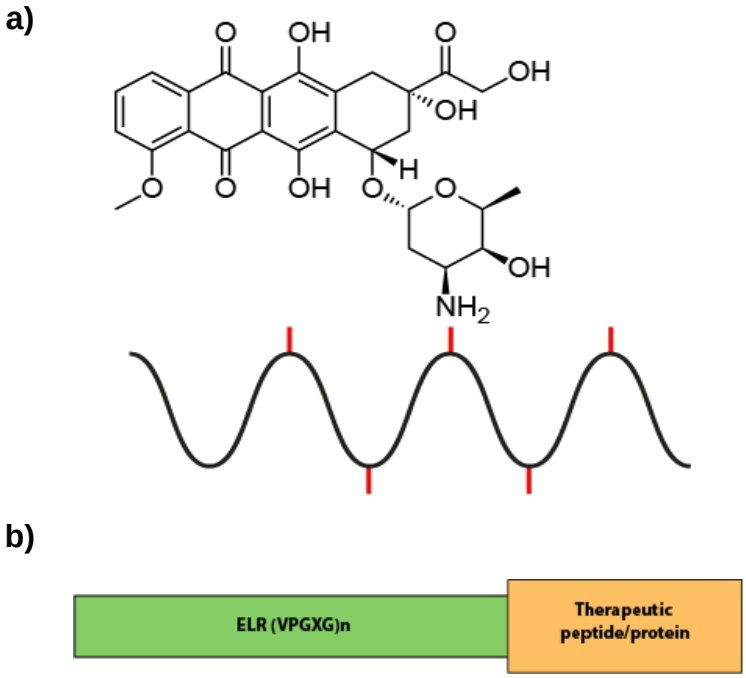

Figure 2. Schematic representation of a) ELR-Dox bioconjugated via lysine or cysteine amino acids (represented as a red line) and b) a therapeutic fusion recombinamer.

2.2.1. Chemical bioconjugates

Different ELRs suitable for covalent bioconjugation to other therapeutic molecules have been designed. In every case, chemical conjugation is achieved by the modification of reactive groups in the side chains of diverse amino acids, such as the thiol group in cysteine or the ε-amino group in lysine, thereby introducing cross-linking sites for different molecules. In this case, a single ELR construct can be used, which means a less time-consuming strategy as no molecular biology techniques have to be applied or purification process optimized. Furthermore, non-protein drugs can also be used in this approach.

One of the most widely used molecules in chemical conjugation to ELRs is the chemotherapeutic agent doxorubicin (Dox). In a first attempt, this drug was conjugated by the modification of an amine group from a lysine amino acid residue in the ELR to obtain a free maleimide group. A doxorubicin-hydrazone complex, which is cleavable because of the acid-

labile nature of this hydrazine bond, was subsequently added to react with the latter and achieve bioconjugation. *In vitro* studies regarding the cytotoxicity and subcellular localization of the conjugated drug showed a similar level of squamous cell carcinoma (FaDu) cell death (less than 10% survival after 72 h of treatment) when comparing the free and the conjugated drugs, but a different localization, with the drug itself mostly being internalized in the nucleus, while the ELR-doxorubicin was dispersed throughout the cytoplasm, thus suggesting a different cell death pathway in both cases.[32] In another experiment, doxorubicin was conjugated via cysteine residues and intracellular cleavage of the drug was achieved by either an acid-labile hydrazine bond [33] or an enzymatic reaction.[34] In this case, a Tat domain, a cationic cell-penetrating peptide (CPP) derived from the trans-activating protein found in HIV-1, was fused to the ELR to enhance cellular uptake, along with the GFLG tetrapeptide linker to act as a substrate for lysosomal cathepsin proteases, thus allowing the release of the drug after endocytosis. The cytotoxicity of this "smart" system was tested *in vitro*, and the results showed that ELR-Dox was cytotoxic towards both non- and Dox-resistant carcinoma cell lines, being able to bypass the P-glycoprotein drug efflux that confers resistance to drugs. The relative resistance (obtained by comparing the IC_{50} values of non- and resistant cell lines) against free Dox was 67.6% in the case of drug-resistant uterine sarcoma cells and 31.9% for breast cancer cells, while these values were 0.9% and 1.4%, respectively, for ELR-Dox.

An alternative approach for the delivery of drugs into target tissues, mainly solid tumours, is to bioconjugate them to ELRs that form aggregates above their transition temperature, said temperature lying between normal body temperature and that in a locally heated region, an approach known as hyperthermia, which could help in the localization of the drug inside the tumour. Two different ELRs have been designed and synthetized with this premise, one with a transition temperature of about 41°C and the other with a transition temperature of more than 55°C, to compare the effect of temperature responsiveness and aggregation, or lack of it, on

tumour stacking. These ELRs were fluorescently labelled and observed by *in vivo* fluorescence videomicroscopy after inoculation into a tumour region in mice. It was seen that the thermoresponsive ELR showed a twofold increase in accumulation in heated tumours compared to the non-responsive ELR, thereby suggesting its potential use in hyperthermia-based drug delivery when conjugated to chemotherapeutic drugs.[35] Similar results were found in another study, where a twofold higher uptake of the labelled hyperthermally aggregated ELR by different carcinoma cell lines when compared to the control soluble one was observed, although some differences in uptake were seen between cell lines. A preferential internalization of particles with a size of around 100 nm was also observed by confocal microscopy and flow cytometry.[36]

As in the case of soluble conjugated ELRs, and taking advantage of the thermal response of some of these polypeptides, doxorubicin has been chemically bioconjugated to different ELRs in order to achieve local accumulation of this chemotherapeutic. Thus, the same system and chemical reaction to that described above, namely Tat-ELP-GFLG-Dox,[34] was used in hyperthermia treatment and found to provide a 20-fold enhancement in the cytotoxicity towards MES-SA uterine sarcoma cells, compared to non-thermal inducement, and a cytoplasmic distribution of the drug. Addition of the Tat sequence permits this higher uptake and efficiency of the drug compared with the values found in previous studies with non-conjugated labelled ELRs (see above).[37] Doxorubicin was also bioconjugated to an ELR including another CPP (SynB1-ELR) via C-terminal cysteines and subsequently released in lysosomes after internalization because of its acid-sensitivity. *In vitro* results in E0771 breast cancer cells showed a cytoplasmic distribution of the labelled drug, while *in vivo* experiments carried out in tumour implanted mice treated with hyperthermia resulted in an increase in the drug half-life, no detectable levels of doxorubicin in the heart (which is one of the most dangerous side effects of the free drug) and complete tumour growth inhibition; this effect was only moderate in the

case of the free drug.[38] A different chemotherapeutic drug used in cancer treatment, known as paclitaxel, which arrests the cell cycle and induces apoptosis, was similarly conjugated to the cell-penetrating peptide fusion ELR SynB1-ELR by coupling of hydrazine-derivitized paclitaxel and a thiol reactive maleimide group from a C-terminal cysteine residue, resulting in improved drug solubility below the transition temperature of the ELR. This construct was further tested *in vitro* by culture with MCF-7 breast cancer cell line, inducing ELR aggregation by hyperthermia, achieving similar results to those obtained with conventional paclitaxel. However, even paclitaxel-resistant MCF-7 cells were killed with this treatment, thus suggesting a way to overcome drug resistance.[39]

2.2.2. Fusion recombinamers

Alternatively, and taking advantage of the enormous potential of recombinant DNA techniques, therapeutic proteins can be fused to an ELR backbone in order to improve the expression, purification and, additionally, delivery and pharmacokinetics thereof.

Many anti-inflammatory proteins, such as antibodies that recognize pro-inflammatory cytokines, have been recombinantly produced fused to ELRs, a method called ELPylation (for elastin-like polypeptide). This approach allows us to take advantage of the ITC purification system and also of the improved stability of the therapeutic protein once in the blood system. Proof-of-concept studies in this regard were carried out with different proteins fused to an ELR in plants, with satisfactory results in terms of expression and purification being obtained.[40, 41] An anti-human TNF was subsequently designed and integrated into an expression system fused to an ELR using this technique. Production was performed in *Nicotiana tabacum* plants and the chimera, which was found to have similar biological bioactivity to the single antibody when tested both in cell cultures *in vitro* and *in vivo*, was purified by several heating and cooling steps. However, a marked improvement was found in terms of serum half-life, which increased from 28 minutes to 11 hours (24-fold).[42] In a similar manner, IL-10[43] and a HIV-neutralizing

antibody[44] were engineered into an ELR carrying vector for expression in tobacco plants and found to have a similar bioactivity to the native proteins. In a similar manner to the way in which whole proteins have been used in the design of fusion therapeutic drugs, peptides have been fused to ELRs to provide the benefits explained above. In one case, AP1, a ligand for the IL-4 receptor that is highly expressed in tumour cells and which amplifies the expression of some anti-apoptotic proteins, thereby preventing drug-induced cancer cell death, was fused to a VPGVG-based ELR containing six repetitions of the peptide amino acid sequence. This chimera was found to bind to the IL-4 receptor *in vitro* and *in vivo* by way of fluorescent labelling of the AP1-ELR, also exhibiting tumour accumulation.[45]

Another recent application, in this case towards treatment of dry eyes, has been developed by fusing a VPGVG-based ELR to lacritin (Lacrt), a protein component of human tears with pro-secretory activity in the lacrimal gland. This construct allows precipitation of the protein and was found to be a good approach to overcoming the limitation of rapid tear turnover when administrating single Lacrt when tested *in vivo*, thus becoming a potential delivery system for the treatment of dry eyes.[46]

As in the case of chemically bioconjugated drug-ELR constructs, fusion recombinamers have also been designed in order to achieve tumour accumulation by hyperthermia by joining ELRs to different tumour-targeting drugs. One of the first attempts in this field involved fusion of a region of c-Myc (H1) known to inhibit c-Myc transcriptional function, together with the cell-penetrating peptide penetratin, to a thermally responsive ELR to enhance cellular uptake. The results showed a twofold increase in the anti-proliferative properties of the thermoresponsive recombinamer in the MCF-7 breast cancer cell line when compared to a control non-aggregated recombinamer after hyperthermia.[47] This same structure was fused to different CPPs to perform a compartment-directed uptake, with maximum cell death being achieved when using Bac, a sequence that directs aggregates to the cell nucleus.[48] Subsequent studies with a very similar

construct were performed using this recombinamer as a therapeutic agent for the treatment of glioblastoma *in vivo* in rats. The results showed an 80% reduction in tumour volume after hyperthermia-induced aggregation, therefore this approach was suggested to be an alternative for drug delivery to brain tumours.[49] However, experiments regarding the cellular uptake efficiency and retention of ELRs in solid tumours after hyperthermia were only performed recently. These studies showed that larger ELRs are better when these properties are taken into account.[50]

Another peptide, in this case derived from lactoferrin (L12), has been fused to a thermally responsive ELR and the Tat domain and tested for the treatment of pancreatic cancer *in vitro*. The results showed a high cytotoxicity of the recombinamer when cultured with MIA PaCa-2 pancreatic adenocarcinoma cells after thermally induced aggregation in comparison with a control non-aggregated fusion ELR.[51] Other studies have used the peptide p21, which arrests the cell cycle, to prevent cell proliferation and therefore as a potential drug for the treatment of cancer. One of the first ELR-p21 recombinamers, which was combined with Bac CPP, showed internalization of ELR aggregates and further anti-proliferative effects by the inhibition of the cell cycle in SKOV-3 ovarian cancer cells and MCF-7 breast cancer ones.[52] An analogous fusion recombinamer was combined with gemcitabine, a chemotherapeutic agent used in the treatment of pancreatic cancer, to enhance the anti-tumour effect while lowering the drug dose to reduce its side effects. The results showed cytotoxicity in MIA PaCa-2 and Panc-1 pancreatic cancer cell lines and tumour growth inhibition in mice *in vivo* after hyperthermia.[53]

3. Drug delivery using ELR-based particles

3.1. Self-assembled ELRs for nano- and microparticle synthesis

The recombinant origin of ELRs allows absolute control over their physical and chemical features, such as functionality, biocompatibility and self-assembly among others.[54-58] This self-

assembly behavior can be induced by the design of the ELR construct itself, conjugation of a drug, addition of charged molecules or mediated by an external stimulus, such as temperature, pH, etc.

The thermo-sensitivity of ELRs makes them ideal for constructs made from different hydrophobic and hydrophilic blocks, constituting what are known as “block copolymers”. For example, in an aqueous solution of a diblock ELR, a dehydration process and decrease in the polarity of this block triggers a conformational change of the whole ELR diblock from unimer to micelle above the transition temperature of the hydrophobic part. Hence, the hydrophobic block is located inside the micelle, thereby providing stability to the complex, whereas the hydrophilic block is positioned outwards in contact with surrounding water. When the temperature is increased above the Tt of the hydrophilic block, it dehydrates and the micelles aggregate. The temperature at which the transition from a soluble ELR diblock to a micelle occurs is called the critical micelle temperature (CMT). This CMT can be controlled by varying the length and composition of the hydrophobic block, whereas the size of the micelle can be controlled by varying the ELR length and the hydrophilic-to-hydrophobic block ratio.[59] The high versatility in the design of ELR diblocks even allows control over whether self-assembly results in micelles or hollow vesicles by varying the block arrangement and length. This conclusion was reached in studies with different ELR constructs comprising glutamic acid and alanine blocks, as described by Martin *et al.*[55] ELR diblocks can be functionalized for different purposes by genetically inserting protein domains into their sequence. Thus, a binding protein such as FKBP (*Plasmodium falciparum* FK506-binding protein), which targets the drug rapamycin, was inserted into the hydrophilic domain of the ELR diblock and used to enhance the affinity of the ELR for this drug **(Figure 3)**. This system was tested *in vitro* in a breast cancer model cell line and was found to increase the loading capacity and release, and therefore the anti-cancer activity, of the drug.[60] Additionally, ELR diblocks were designed with the knob

protein domain from adenovirus to improve the cellular internalization of the complex. The knob domain binds to the overexpressed adenovirus receptor (CAR), which is widely located in hepatocytes and acinar cells. Formation of the ELR micelle above a certain Tt, with a size of about 40 nm, caused the knob domains to be exposed outwards. The addition of protein domains to the ELR diblock facilitated its interaction and internalization inside the hepatocytes compared with the non-modified ELR.[61]

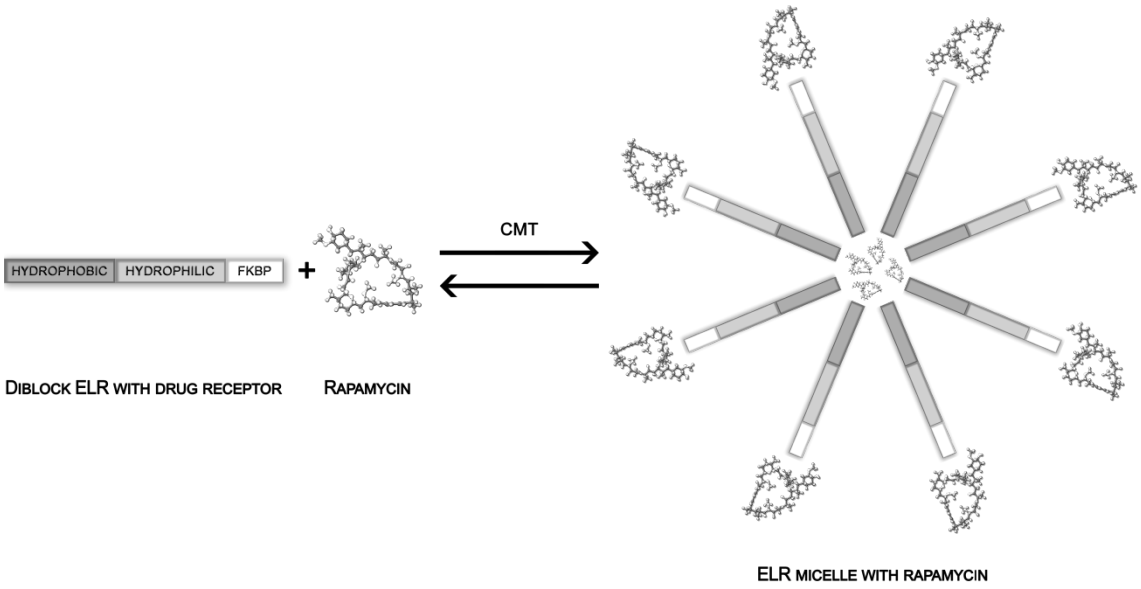

Figure 3. ELR di-block functionalized with FKBP. FKBP was bound at the C-t by genetic engineering techniques. The diblock binds to rapamycin with high affinity via the binding protein FKBP. Above the CMT the diblock adopts its micelle conformation, increasing the loading capacity for rapamycin and exposing it on the surface.

Self-assembly of ELRs can also be obtained chemically by cross-linking methods, forming thermo-responsive microgel capsules (MCs) that can be used for drug-delivery purposes. Chemical cross-linking maintains the shape of the microspheres while the pore size can be reversibly changed by the application of external stimuli, thus controlling drug delivery. Na *et al.*, for example, used a mixture of ELR and albumin at different mass ratios and cross-linked the ELR to both enhance shape stability and control the structure of the surface when changing

the temperature.[62] Above the Tt of the ELRs tested the release of albumin and prednisone acetate as model drugs increased due to the formation of a porous structure; however, they were released slowly below this Tt. Gradual release was found in the temperature range 20–40°C. Subsequently, Cheng *et al.* created ELR/BSA MCs using a two-step cross-linking method[63] and evaluated the ability of these MCs to release a drug using two model molecules with similar molecular weights, namely rhodamine B and the negatively charged FITC. The results showed a charge-independent release of both molecules from the microcapsules. Additionally, the high loading and release speed of the cargo molecules was confirmed upon increasing the temperature above the Tt of the ELR. In contrast, narrowing of the pores, and therefore no release of the cargo was observed when decreasing the temperature below the Tt. Both studies show the potential of ELR microcapsules self-assembled using cross-linking methods and their ability to encapsulate and release drugs in response to temperature changes.

In addition, the biocompatible nature of ELRs means that they have been used in the formation of microparticles following layer-by-layer strategies for the encapsulation and delivery of a model cargo protein into cells. Thus, for example, a layer-by-layer approach was employed to create microparticles made from alginate-chitosan and chitosan-ELR as a proof-of-concept for the methodology.[64] In a more recent work, multilayered coated capsules made of chitosan and an ELR functionalized with bioactive sequences were used as building blocks and assembled on spherical particles of calcium carbonate.[65] The ELR was designed to contain the RGD sequence, thereby improving recognition of the particle by cell adhesion integrins. The resulting multilayered particles, with a size of about 3-4 μm, loaded with the model protein ovoalbumin were incubated with hMSCs for 72 hours. After this time, non-cytotoxic effects were observed. Additionally, degraded DQ-ovoalbumin, which was used to monitor the fluorescence changes in the cargo, showed internalization and higher degradation when encapsulated in RGD-functionalized ELR-chitosan microparticles and was found to be located mainly in the

cytoplasm **(Figure 4)**. This strategy paves the way to the creation of protein-based drug-delivery architectures made from ELRs, thereby conferring versatile design alternatives and highly biocompatible properties.

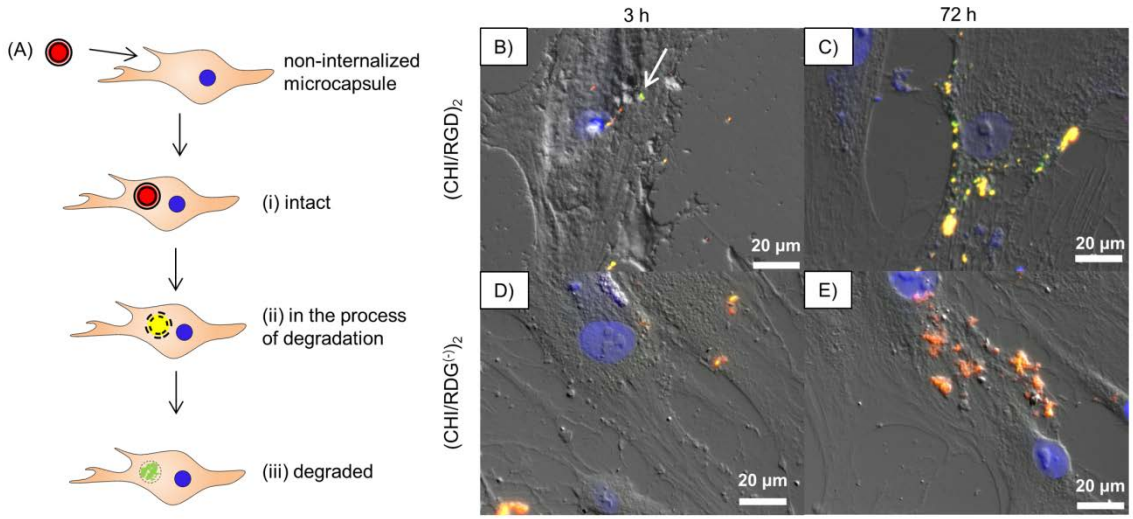

Figure 4. (A) Degradation stages of DQ-ovalbumin inside the cell. Fluorescence and DIC microscopy images of (CHI/ELR-RGD)$_2$ and (CHI/ELR-RDG(−))$_2$ microcapsules loaded with DQ-ovalbumin incubated with hMSCs for (B and D) 3h or (C and E) 72h and DAPI staining. The arrow in B indicates a bright green spot. A shift from red/orange to green/yellow indicates a transition from a fully intact to a degraded cargo.

Self-assembly can also be triggered by the covalent attachment of hydrophobic drugs, such as doxorubicin or paclixatel.[66, 67] Thus, in Chilkoti's group, a high molecular weight ELR was conjugated to doxorubicin via the cysteine residues genetically incorporated at one end of the polypeptide. The hydrophobic drug triggered self-assembly of the recombinamer into nanoparticles with a diameter of 40nm in which the drug was located at the core and surrounded by the hydrophilic ELR. *In vivo* assays with a colon cancer mouse model showed the induction of almost complete tumor regression, with a fourfold higher maximum tolerated dose than for the free drug after a single administration and promoted by the ELR nanoparticles.[67]

The addition of charged molecules, such as nucleic acids, in a non-covalent manner can also induce the self-assembly process. Thus, negatively charged plasmid DNA can electrostatically interact with a specifically designed ELR for use in gene delivery. In one example, an ELR containing a cationic oligolysine tail (K8-ELP (1-60)) was complexed with pDNA, with the results showing a good complexation ability and stability of the polyplexes for the N/P ratios tested. In terms of transfection, ELR-pDNA polyplexes with a size of about 115nm and encoding for the GFP (green fluorescent protein) were able to transfect the MCF-7 cells tested, although some cytotoxic effects were observed,[68] probably due to the oligolysine tail. A recent study of ELRs designed with the functional motifs named penetratin, LAEL fusogenic peptide and imidazole groups showed the formation of stable polyplexes of around 200nm of size and +24mV of zeta potential. Additionally haemocompatibility and cytotoxicity assays exhibited the innocuous nature of these ELRs. The ELR containing the LAEL peptide resulted the best option for cellular uptake and transfection among the ELRs tested.[69] These results demonstrated the potential of ELR for gene therapy applications.

Finally, the aforementioned external temperature stimulus can trigger the self-assembly of ELRs by virtue of their smart nature: when the temperature increases, aggregation phenomena in ELRs lead to a spontaneous coacervation process. ELRs in the coacervation phase can be exploited and used as carriers for prolonged drug delivery. In this regard, studies with a KGF-ELR fusion polymer (KGF is keratinocyte growth factor) suggested the possible use of this polymer for the treatment of chronic wounds, with coacervation from the ELR component occurring at 37°C even when the growth factor was bound to the ELR. If the coacervate was located in the damaged tissue, this would allow the progressive administration of KGF, thereby enhancing granulation and re-epithelization when compared with a fibrin gel containing KGF in genetically diabetic male mice.[70] Alternatively, *in vitro* assays with C2C12 cells showed that another type of ELR containing $(VPAVG)_{220}$, which formed nanoparticles with a size of 237nm,

was able to encapsulate significant amounts of both BMP-2 and BMP-14 (bone morphogenetic proteins). In this case, the release process *in vitro* was associated with a two-phase profile characterized by a burst delivery during the first 24 hours and followed by a sustained and slower release over 14 days.[71] This slow release of both KGF and BMP factors suggests the significant potential of ELR coacervates for both tissue regeneration applications and for the release of other types of molecules, such as therapeutic drugs.

Alternatively, self-assembled ELRs have been used as nanovaccines for tuberculosis disease.[72] The incorporation of an antigenic molecule from *M. tuberculosis* at the hydrophilic terminus of the ELR diblock $E_{50}I_{60}$ (comprising a hydrophilic block based on glutamic acid as guest residue (E_{50}) and a hydrophobic block based on isoleucine (I_{60})) using recombinant techniques resulted in the formation of Ag-$E_{50}I_{60}$. This construct was found to reversibly self-assemble into highly biocompatible, multivalent, monodisperse and stable nanoparticles. These particles were able to trigger an innate immune response following by an adaptative Th2 response due to the presence of IL-5 and up-regulation of IgM and IgG in *in vivo* assays. The control used (diblock ELR or Ag alone) did not trigger any immunomodulatory response. These results support the use of this kind of ELR di-block construct as a potential antigen carrier for the development of more effective vaccines.

3.2. Self-assembled ELR particles and hyperthermia

The thermal sensitivity provided by ELRs materials is a very attractive feature from the point of view of cancer therapy as the tumor can be heated externally and, consequently, the ELR can trigger a phase transition in the damaged tissue locally.

Tumor tissues are different to normal tissues, with components such as tumor vessels having been reported to possess larger intercellular pores than unaffected vessels. Additionally, a greater accumulation of lipids and macromolecules from the tumor cells occurs in cancer due

to restricted lymphatic drainage. This phenomenon, known as the enhanced permeability and retention (EPR) effect, together with the local application of mild-hyperthermia (40-44°C)[73] as adjuvant treatment for cancer therapy, can be taken advantage of by using thermosensitive materials such as ELRs. ELR diblocks comprising a hydrophobic block with a Tt > 42°C that show a self-assembly behavior from unimer to micelle when local mild-hyperthermia conditions are applied have been previously studied as regards their physical properties and *in vitro* behavior. The incorporation of different functional motifs has been shown to maintain the natural behavior of ELR diblocks and improve their action.[60, 61] By way of illustration, when the RGD cell-adhesion motif was included into an ELR sequence comprising a hydrophilic block (containing valine, glycine, and alanine as guest residues in a 1:7:8 ratio) and a hydrophobic block (valine), the self-assembly temperature was maintained.[74] Additionally, an effect known as multivalency of the RGD was observed in the micelle conformation in response to the external application of hyperthermia, thereby constituting a "tunable thermal switch". This multivalency favors the uptake and binding of ELR diblocks, especially RGD-ELR-64/90, in the K562 leukemia cells tested.

Other ELR diblock constructs based on the same amino-acid sequence mentioned above, but differing in the hydrophilic and hydrophobic block lengths, were fused to two single domain proteins, namely thioredoxin (Trx) and a fibronectin type III domain (Fn3) that binds the $\alpha_v\beta_3$ integrin. Binding of these domain proteins did not alter the micelle formation triggered by the ELR, and the resulting ELR diblocks functionalized with either Trx or Fn3 exhibited a size of 24-37 nm. One of these diblocks, named ELR-96-90, which comprises a hydrophilic block of 96 pentapeptides and a hydrophobic block of 90 pentapeptides fused to Fn3, showed bioactivity, enhancing targeting and uptake by integrin-overexpressing K562/$\alpha_v\beta_3$ cells.[75] Additionally, the design of more complex self-assembled systems for drug delivery under mild hyperthermia conditions was also attempted. To this end, ELR diblocks functionalized with cell-penetrating

peptides (CPP), as internalization motifs, and a drug payload were designed and named as "nanopeptifiers".[76] The construct was genetically engineered from the BH3 peptide (derived from the proapoptotic Bak protein) located at the N-terminal end separated by a RVRR peptide acting as linker to the ELR diblock and followed by the CPP at the C-terminus (named as Arg8-ELR_{BC}–cBH3). In this manner, the BH3 peptide load was sequestered in the micelle core upon self-assembly. The RVRR peptide was chosen due to its ability to be cleaved by furin and cathepsin B proteases, thus allowing intracellular release of the peptide drug following endocytic uptake. The diblock self-assembled from the unimer to the micelle under mild hyperthermia conditions (42°C), with the CPPs being located on the outer surface of the micelle in high density. The tunable switching ability of CPP density in ELR diblock constructs had already been evaluated in earlier studies.[77] The cellular uptake confirmed the tunable nature of intracellular delivery by thermally triggered CPP-ELR diblock micelle assembly and release of the BH3 peptide. The bioactivity was assessed by activation of caspase-3 involved in the apoptosis processes. Thus, when the proapoptotic peptide was present in the nanopeptifier construct, the platform provided a cytotoxic switch inducing apoptosis only when the ELR diblock was self-assembled.

In addition to diblocks designed for hyperthermia approaches, ELR coacervates have also been applied in *in vivo* assays for this purpose. Thus, an injectable and thermally responsive ELR bound to ^{131}I radionuclide was evaluated in terms of tumor retention and antitumor efficacy.[78] The results showed a 40% tumor retention for the ELR bound to the radionucleide, thus providing the added advantage of improving the efficacy of the radioisotope due to its retention as a result of ELR aggregation.

In summary, ELRs are stimulating materials of significant interest for biomedical applications as their recombinant nature allows total control over the architecture and self-assembly features, thus allowing the introduction of sensitivity to external stimulus such as temperature. Novel

applications in cancer therapy, including thermotherapy, are excellent examples of the use of this kind of naturally inspired biomaterials.

4. HYDROGELS AND DEPOTS FOR DRUG DELIVERY

Controlled-release systems usually require the use of a support or scaffold that acts as a drug reservoir, thus allowing sustained release of the therapeutic agent and avoiding "peak and valley" profiles inside the body.[79] Peptide-based hydrogels are gaining increasing attention for drug-delivery purposes thanks to the wide range of design possibilities conferred by the almost infinite number of possible combinations of amino acids. In other words, the structural features and functionalities of the gel network can be easily tailored by carefully engineering the peptide sequence.[80, 81] Within this framework, elastin-based hydrogels are promising candidates for use in the construction of drug-delivery devices because of their protein-based nature and the inherent properties displayed by this class of materials, as highlighted in the introduction. Thus, appropriately designed ELRs display a sol state below body temperature (37°C), forming a viscous coacervate (depot) or hydrogel when implanted in the body. This ability to form a liquid-like state below Tt makes mixing with therapeutic agents efficient and extremely simple. Furthermore, implantation is painless and application is not restricted to accessible areas. Moreover, the injection can be applied directly at the target site, thus helping to extend local drug exposure while minimizing systemic side effects.[82] The protein nature of many pharmaceuticals paves the way for their inclusion into ELR-based devices both inside the ELR depot and bound to the ELR molecule.[83, 84] In addition to their utility as drug reservoirs, ELR have further roles, such as macromolecular carriers, and it has even been speculated that they may act as a shield, protecting the therapeutic agent against protease attack.[85]

With regard to their ability to act as a macromolecular carrier, a system comprising a glucagon-like peptide-1 (GLP-1) fused to an ELR has been developed to achieve glucose control and

therefore potential applications in the treatment of diabetes. GLP-1 is a peptide drug with glucose-dependent insulinotropic effects, thereby helping to avoid the potential risk of hypoglycemia associated with insulin use. However, its rapid plasma clearance and enzymatic degradation have hampered the possibility of its use as a real option for the treatment of diabetes. In order to overcome these difficulties and move towards its future clinical use, Chilkoti's group has developed fusion constructs between an ELR and GLP-1 in which the ELR plays an active role by allowing depot formation after injection and also acts as a long-circulating macromolecular carrier.[86] Thus, GLP-1-ELR fusions were demonstrated to be proteolytically more stable than the GLP-1 monomer, probably due to a steric effect of the ELR, which hinders access of proteases to their cleavage site. Moreover, *in vivo* studies conducted in a mouse model showed that (GLP-1)-ELR depot displays an extended temporal reduction of glucose levels by approximately 30% for 5 days. In contrast, both controls, namely (GLP-1)-ELR monomer and GLP-1, resulted in a sharp and non-sustained drop in glucose levels.

Using a similar approach, a (GLP-1)-ELR depot was designed so that GLP-1 is released in a monomeric form (without an ELR).[87] In this design, GLP-1 oligomers are flanked by protease cleavage sites and fused to the ELR such that cleavage at these sites allows GLP-1 release from the depot. The resulting depot was termed a "protease operated depot" (POD). A single injection of GLP-1-POD was subsequently shown to reduce blood glucose levels for up to five days in mice.

As stated above, injection of a pharmaceutical device directly into the target tissue minimizes the side effects on healthy tissues as well as allowing complicated locations that are not easily accessible by systemic administration to be reached. A good example of a body location that is not easily reached is the joints, since their avascular nature complicates access via systemic administration. In this sense, intra-articular injection of ELR-depots has been explored as a feasible option to achieve controlled drug delivery in such locations. Thus, biodistribution of

an ELR after injection into the knee joint compartment in a rat model demonstrated that the ability of the ELR to aggregate prolonged the half-life of the polymer by more than 25-fold when compared to a soluble non-aggregating ELR.[88]

A further step in the application of this kind of ELR-depot in the treatment of joint diseases, such as osteoarthritis, involved the construction of a fusion construct between an ELR and IL-1Ra (interleukin-1 receptor antagonist).[89] Although the interaction of IL-1Ra fused to the ELR moieties with IL-R was slower when compared to the free IL-1Ra, such an approach represents an appropriate strategy for prolonging the presence of bioactive therapeutic agents following intra-articular delivery.

When immunosuppression treatments are required, the possibility of local delivery becomes a must to avoid systemic exposure. Thus, a tritium-radiolabeled ELR (depot and soluble) has been injected overlying the L5 dorsal root ganglion of rats, with the resulting biodistribution data clearly showing an enhanced half-life (sevenfold longer) of ELR depot in the perineural space when compared to the soluble ELR along with a 14-fold reduction in systemic exposure.[90] This study opens the way to the use of ELR depots as effective devices for the release of immunomodulating therapeutic agents to treat local neuroinflammation. Another striking approach related to the use of ELR depots in neuroinflammation treatment involved the development of an injectable depot in which curcumin (a TNFα antagonist) was conjugated to the ELR via a degradable carbamate linkage.[91] Intramuscular injection of the resulting curcumin-ELR depot proximal to the sciatic nerve in mice resulted in a fivefold higher level of the drug at 96h as compared to the free drug while limiting systemic exposure (sevenfold reduction).

Radiation therapy is a widely used strategy for the treatment of tumors. Motivated by the development of a radionuclide carrier that is able to prevent dissemination from the injection site, an ELR conjugated with iodine-131 depot has been constructed.[78] The effectiveness of this

depot arises due to some of the particular characteristics of radionuclide therapy. For example, drug release from the ELR carrier is not necessary. Moreover, radionuclide emissions can kill tumor cells from a distance (e.g. the penetration distance of ^{131}I beta emission is 910 μm), so no internalization by tumor cells is required. In this approach, although a significant tumor growth delay was observed, complete regression still required some optimization in terms of ELR design, concentration and infusion protocol. This optimization was subsequently achieved thanks to a meticulous and systematic study of the physical properties of the ELR and their influence on tumor retention and the concomitant translation into a therapeutic advantage.[92] Thus, the optimized ^{131}I-ELR depot delayed tumor growth in 100% of the tumors in two human xenografts (FaDu and PC-3) and cured more than 67% of tumor-bearing animals.

The characteristic hydrophobic associations of ELRs have been exploited in the aforementioned examples to achieve physically cross-linked depots or hydrogels. However, non-covalent bonding usually results in suboptimal mechanical properties. In order to overcome this issue, Chilkoti's group has developed hydrogels with properties intermediate between those of coacervates and chemical hydrogels by engineering the presence of cysteine residues along the ELR structure.[93] The resulting hydrogels display rapid gelation under mild oxidative conditions and their *in vivo* intratumoral administration to nude mice bearing human pharynx squamous xenografts showed enhanced tumor retention when compared to their control (ELR without cysteine), in addition to an ideal homogeneous distribution across the entire tumor.

One remarkable strategy for obtaining entirely physical and stable ELR-based hydrogels is to incorporate bioinspired peptide motifs other than elastin along the ELR backbone. One interesting example of bio-inspired peptide motifs for physical crosslinking purposes is the use of silk fibroin domains. Such domains, the sequence of which follows patterns such as GAGAGS, are known to adopt an anti-parallel β-sheet structure characterized by high stability and irreversibility. Such properties are maintained when GAGAGS motifs are engineering

along an ELR molecule, giving rise to so-called SELR (silk-elastin like recombinamer). Many SELR-based hydrogels have been reported in the literature[94-97] and some of them have been successfully explored for drug-delivery purposes.[98-101] Indeed, the utility of SELRs has been explored in both the field of drug delivery and for the delivery of viral vectors. Specifically, an adenovirus containing both thymidine kinase-1 and luciferase genes has been incorporated into an SELR matrix (SELP 815K hydrogel) and the resulting system injected intratumorally into a nude mice model of head and neck cancer.[102] The bioluminescence provided by the luciferase gene was used to determine gene transfection efficiency, duration of transgene expression and biodistribution. It was clearly shown that administration within the SELP 815K hydrogel resulted in greater confinement of the therapeutic agent at the tumor site with no evidence of any spreading to the liver, thereby contrasting with the results obtained for the saline/adenovirus treatment group, in which 50% of animals showed clear liver dissemination. As regards anticancer efficacy, an up to fivefold reduction in tumor volume was observed in the adenovirus-SELP 815K group when compared to the saline/adenovirus treatment group. In a further design step, an advanced version characterized by the presence of a matrix-metalloproteinase (MMP) sequence along the SELP 815K was constructed.[103] The MMP-sensitivity of these hydrogels suggests that they could potentially respond to local changes in these proteases when injected intratumorally.

In addition to the aforementioned approaches, in which the self-assembly behavior of elastin-based constructs is exploited to form depots and hydrogels, another approach for achieving and maintaining a three-dimensional ELR-based scaffold involves the inclusion of an amino acid that can subsequently be modified to contain chemical groups for covalent cross-linking.[104] Such strategies usually involve incorporation of the lysine residue “K” at the “X” position of some of the (VPGXG) pentapeptides present in the ELR. For example, ELRs have been chemically modified at their lysine amino acids to bear the reactive groups required for “click

chemistry" reactions, namely azide and cyclooctyne. Since the chemically reactive groups are incorporated into the ELR structure prior to the crosslinking reaction, there is no risk of the release of excess crosslinking reagents. Furthermore, subsequent covalent crosslinking takes place under mild physiological conditions and with short reaction times.[105, 106] Such an approach would permit the homogeneous entrapment of drugs and biomolecules inside the hydrogel, while guaranteeing their biocompatibility. In a further step, the Click-ELR hydrogels reported in [106] have been used to coat vascular stents **(Figure 5)**,[107] thus paving the way to the next generation of stents with drug-release properties. Another approach for obtaining high selectivity involves taking advantage of the specificity achieved by enzymatic reactions, such as that catalyzed by transglutaminase.[108] For example, ELR hydrogels crosslinked via transglutaminase have been engineered so that exposure to the proteolytic activity derived from *P. aeruginose* and human polymorphonuclear leukocytes triggers the release of a model compound from the matrix.[109] The final goal of this kind of approach, in which the hydrogel is engineered to be sensitive to specific proteases, is to obtain a vehicle that can sense a chemical abnormality in damaged tissue and release a therapeutic agent in response to it. Thus, the well-stablished correlation between many relevant pathological conditions and elastolytic conditions could potentially allow the sustained release of drugs that ultimately interfere with the pathological process that triggered its release.

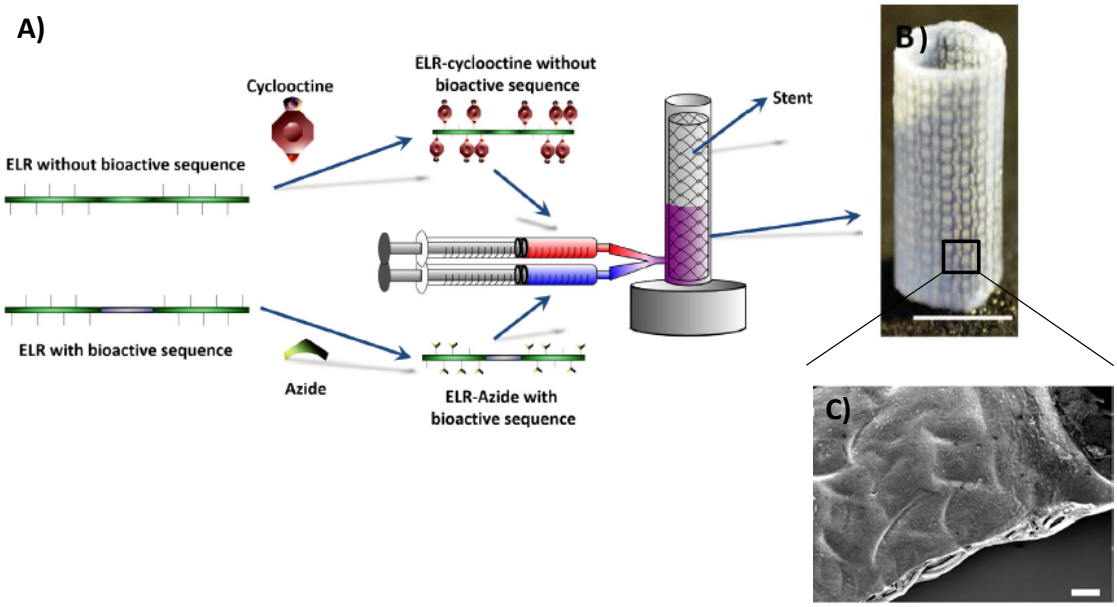

Figure 5. A) Schematic representation of the molding procedure of Click-ELR-coated stents. B) Warp-knitted nitinol stent coated with Click-ELR. C) SEM image of a Click-ELR stent.[107]

The above examples reflect the strong commitment of the scientific community to the development of novel and advanced drug-delivery depots and hydrogels that are able to meet specific therapeutic needs. Although it is clear that there have been several significant breakthroughs in this field, there is still a long way to go to achieve the ultimate goal, namely to create devices that are able to act as the body does by detecting where, when and how they should release their own biological compounds.

5. CONCLUSIONS

There is currently an increasing need for new pharmacological platforms that can release their therapeutic agents in a well-controlled spatiotemporal manner. Additionally, this goal must be accomplished in the complex environment generated by living tissues, bodily fluids and/or even inside cells. As such, these delivery systems must be intrinsically complex as they need to address the multiple challenges that living systems will generate during their action. In this sense, ever more sophisticated materials are needed in order to achieve truly efficient and

advanced drug-delivery systems. Among the many options currently being explored, protein-based polymers and, in particular, elastin-like recombinamers stand out due to the possibility of incorporating virtually any peptide-function present (or not) in any natural protein into their composition, thus making these materials a source of therapeutic agents on their own. However, they may also be excellent carriers for delivering drugs to a designated target tissue or even intracellular location. Once again, the advantage of incorporating functional peptides that can help to protect the borne therapeutic agent and precisely deliver it to its final destination within their peptide sequence is a remarkable advantage arising from the use of these kinds of materials. The combination of biofunctionality and an ability to self-assemble, which can easily be combined and exploited in such protein-based materials, is unique. Additionally, as these materials are produced using a purely synthetic gene, genetic-engineering approaches can applied in an essentially unrestricted manner to ensure that the final composition is only dictated by these engineering designs. All this, along with the other additional advantages described above, has already resulted in therapeutic platforms with a markedly increased efficacy. However, since the potential of this approach is far from being fully explored, this is just the beginning, and the next few years will witness the development and clinical application of sophisticated ELR-based therapeutic platforms that will open up the way to therapeutic strategies that are currently beyond our reach.

AUTHOR INFORMATION

Corresponding Author
*E-mail: roca@bioforge.uva.es Fax: +34 983 184698 Tel: +34 983 184585

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful for ERDF funding from the EU and MINECO (PRI-PIBAR-2011-1403, MAT2012-38043, MAT2013-42473-R and MAT2013-41723-R), the JCyL (projects VA152A12, VA155A12 and VA313U14), the CIBER-BBN, the JCyL and the Instituto de Salud Carlos III under the "Network Center of Regenerative Medicine and Cellular Therapy of Castilla and Leon"

REFERENCES

(1) Allen, T. M., and Cullis, P. R. (2004) Drug Delivery Systems: Entering the Mainstream. *Science 303*, 1818-1822.
(2) Langer, R. (1990) New methods of drug delivery. *Science 249*, 1527-33.
(3) Price, R., Poursaid, A., and Ghandehari, H. (2014) Controlled release from recombinant polymers. *J Control Release 190*, 304-13.
(4) Macewan, S. R., and Chilkoti, A. (2010) Elastin-like polypeptides: Biomedical applications of tunable biopolymers. *Biopolymers 94*, 60-77.
(5) MacEwan, S. R., and Chilkoti, A. (2014) Applications of elastin-like polypeptides in drug delivery. *J Control Release 190*, 314-30.
(6) Rodriguez-Cabello, J. C., Gonzalez de Torre, I., and Pinedo, G. (2013) CHAPTER 19 Elastin-like Hydrogels and Self-assembled Nanostructures for Drug Delivery, in *Smart Materials for Drug Delivery: Volume 2* pp 180-198, The Royal Society of Chemistry.
(7) Rodriguez-Cabello, J. C., Girotti, A., Ribeiro, A., and Arias, F. J. (2012) Synthesis of genetically engineered protein polymers (recombinamers) as an example of advanced self-assembled smart materials, in *Nanotechnology in Regenerative Medicine* (Navarro, M., and Planell, J. A., Eds.) pp 319, Humana Press.
(8) Arias, F. J., Santos, M., Fernández-Colino, A., Pinedo, G., and Girotti, A. (2014) Recent Contributions of Elastin-Like Recombinamers to Biomedicine and Nanotechnology. *Current Topics in Medicinal Chemistry 14*, 819-836 (18).
(9) Girotti, A., Fernandez-Colino, A., Lopez, I. M., Rodriguez-Cabello, J. C., and Arias, F. J. (2011) Elastin-like recombinamers: biosynthetic strategies and biotechnological applications. *Biotechnol. J. 6*, 1174-86.
(10) Rodriguez-Cabello, J. C., Pierna, M., Fernandez-Colino, A., Garcia-Arevalo, C., and Arias, F. J. (2011) Recombinamers: combining molecular complexity with diverse bioactivities for advanced biomedical and biotechnological applications. *Adv Biochem Eng Biotechnol 125*, 145-79.
(11) Urry, D. W. (1993) Molecular Machines - How Motion and Other Functions of Living Organisms Can Result from Reversible Chemical-Changes. *Angew. Chem., Int. Ed. Engl. 32*, 819-841.

(12) Urry, D. W. (2006) *What sustains life? Consilient mechanisms for protein-based machines and materials*, Springer-Verlag, New York.
(13) Venkatachalam, C. M., and Urry, D. W. (1981) Development of a linear helical conformation from its cyclic correlate. β-Spiral model of the elastin poly(pentapeptide) (VPGVG)n. *Macromolecules 14*, 1225-1229.
(14) Urry, D. W. (1997) Physical Chemistry of Biological Free Energy Transduction As Demonstrated by Elastic Protein-Based Polymers. *Journal of Physical Chemistry B 101*, 11007-11028.
(15) Li, B., Alonso, D. O. V., and Daggett, V. (2001) The molecular basis for the inverse temperature transition of elastin. *Journal of Molecular Biology 305*, 581-592.
(16) Stuart, M. A., Huck, W. T., Genzer, J., Muller, M., Ober, C., Stamm, M., Sukhorukov, G. B., Szleifer, I., Tsukruk, V. V., Urban, M., Winnik, F., Zauscher, S., Luzinov, I., and Minko, S. (2010) Emerging applications of stimuli-responsive polymer materials. *Nat Mater 9*, 101-13.
(17) Nikolov, Z. L., and Woodard, S. L. (2004) Downstream processing of recombinant proteins from transgenic feedstock. *Current opinion in biotechnology 15*, 479-86.
(18) Osicka, R., Prochazkova, K., Sulc, M., Linhartova, I., Havlicek, V., and Sebo, P. (2004) A novel "clip-and-link" activity of repeat in toxin (RTX) proteins from gram-negative pathogens. Covalent protein cross-linking by an Asp-Lys isopeptide bond upon calcium-dependent processing at an Asp-Pro bond. *The Journal of biological chemistry 279*, 24944-56.
(19) Liu, W. J., Wu, Q., Xu, B., Zhang, X. Y., Xia, X. L., and Sun, H. C. (2014) Single-step purification of recombinant proteins using elastin-like peptide-mediated inverse transition cycling and self-processing module from Neisseria meningitides FrpC. *Protein expression and purification 98*, 18-24.
(20) Perler, F. B., Davis, E. O., Dean, G. E., Gimble, F. S., Jack, W. E., Neff, N., Noren, C. J., Thorner, J., and Belfort, M. (1994) Protein splicing elements: inteins and exteins--a definition of terms and recommended nomenclature. *Nucleic acids research 22*, 1125-7.
(21) Wood, D. W., Wu, W., Belfort, G., Derbyshire, V., and Belfort, M. (1999) A genetic system yields self-cleaving inteins for bioseparations. *Nature biotechnology 17*, 889-92.
(22) Banki, M. R., Feng, L., and Wood, D. W. (2005) Simple bioseparations using self-cleaving elastin-like polypeptide tags. *Nature methods 2*, 659-61.
(23) Fong, B. A., and Wood, D. W. (2010) Expression and purification of ELP-intein-tagged target proteins in high cell density E. coli fermentation. *Microbial cell factories 9*, 77.
(24) Fong, B. A., Gillies, A. R., Ghazi, I., LeRoy, G., Lee, K. C., Westblade, L. F., and Wood, D. W. (2010) Purification of Escherichia coli RNA polymerase using a self-cleaving elastin-like polypeptide tag. *Protein science : a publication of the Protein Society 19*, 1243-52.
(25) Hu, F., Ke, T., Li, X., Mao, P. H., Jin, X., Hui, F. L., Ma, X. D., and Ma, L. X. (2010) Expression and purification of an antimicrobial peptide by fusion with elastin-like polypeptides in Escherichia coli. *Applied biochemistry and biotechnology 160*, 2377-87.
(26) Hara, S., and Yamakawa, M. (1996) Production in Escherichia of moricin, a novel type antibacterial peptide from the silkworm, Bombyx mori. *Biochemical and biophysical research communications 220*, 664-9.
(27) Gerashchenko, O. L., Zhuravel, E. V., Skachkova, O. V., Khranovska, N. N., Filonenko, V. V., Pogrebnoy, P. V., and Soldatkina, M. A. (2013) Biologic activities of recombinant human-beta-defensin-4 toward cultured human cancer cells. *Experimental oncology 35*, 76-82.

(28) Shen, Y., Ai, H. X., Song, R., Liang, Z. N., Li, J. F., and Zhang, S. Q. (2010) Expression and purification of moricin CM4 and human beta-defensins 4 in Escherichia coli using a new technology. *Microbiological research 165*, 713-8.
(29) Lim, D. W., Trabbic-Carlson, K., Mackay, J. A., and Chilkoti, A. (2007) Improved non-chromatographic purification of a recombinant protein by cationic elastin-like polypeptides. *Biomacromolecules 8*, 1417-24.
(30) Xu, X., Jin, F., Yu, X., Ji, S., Wang, J., Cheng, H., Wang, C., and Zhang, W. (2007) Expression and purification of a recombinant antibacterial peptide, cecropin, from Escherichia coli. *Protein expression and purification 53*, 293-301.
(31) Yang, K., Su, Y., Li, J., Sun, J., and Yang, Y. (2012) Expression and purification of the antimicrobial peptide cecropin AD by fusion with cationic elastin-like polypeptides. *Protein expression and purification 85*, 200-3.
(32) Dreher, M. R., Raucher, D., Balu, N., Michael Colvin, O., Ludeman, S. M., and Chilkoti, A. (2003) Evaluation of an elastin-like polypeptide-doxorubicin conjugate for cancer therapy. *J Control Release 91*, 31-43.
(33) Furgeson, D. Y., Dreher, M. R., and Chilkoti, A. (2006) Structural optimization of a "smart" doxorubicin-polypeptide conjugate for thermally targeted delivery to solid tumors. *J Control Release 110*, 362-9.
(34) Bidwell, G. L., 3rd, Davis, A. N., Fokt, I., Priebe, W., and Raucher, D. (2007) A thermally targeted elastin-like polypeptide-doxorubicin conjugate overcomes drug resistance. *Investigational new drugs 25*, 313-26.
(35) Meyer, D. E., Kong, G. A., Dewhirst, M. W., Zalutsky, M. R., and Chilkoti, A. (2001) Targeting a genetically engineered elastin-like polypeptide to solid tumors by local hyperthermia. *Cancer research 61*, 1548-54.
(36) Raucher, D., and Chilkoti, A. (2001) Enhanced uptake of a thermally responsive polypeptide by tumor cells in response to its hyperthermia-mediated phase transition. *Cancer research 61*, 7163-70.
(37) Bidwell, G. L., 3rd, Fokt, I., Priebe, W., and Raucher, D. (2007) Development of elastin-like polypeptide for thermally targeted delivery of doxorubicin. *Biochemical pharmacology 73*, 620-31.
(38) Moktan, S., Perkins, E., Kratz, F., and Raucher, D. (2012) Thermal targeting of an acid-sensitive doxorubicin conjugate of elastin-like polypeptide enhances the therapeutic efficacy compared with the parent compound in vivo. *Molecular cancer therapeutics 11*, 1547-56.
(39) Moktan, S., Ryppa, C., Kratz, F., and Raucher, D. (2012) A thermally responsive biopolymer conjugated to an acid-sensitive derivative of paclitaxel stabilizes microtubules, arrests cell cycle, and induces apoptosis. *Investigational new drugs 30*, 236-48.
(40) Conley, A. J., Joensuu, J. J., Jevnikar, A. M., Menassa, R., and Brandle, J. E. (2009) Optimization of elastin-like polypeptide fusions for expression and purification of recombinant proteins in plants. *Biotechnology and bioengineering 103*, 562-73.
(41) Patel, J., Zhu, H., Menassa, R., Gyenis, L., Richman, A., and Brandle, J. (2007) Elastin-like polypeptide fusions enhance the accumulation of recombinant proteins in tobacco leaves. *Transgenic research 16*, 239-49.
(42) Conrad, U., Plagmann, I., Malchow, S., Sack, M., Floss, D. M., Kruglov, A. A., Nedospasov, S. A., Rose-John, S., and Scheller, J. (2011) ELPylated anti-human TNF therapeutic single-domain antibodies for prevention of lethal septic shock. *Plant biotechnology journal 9*, 22-31.

(43) Kaldis, A., Ahmad, A., Reid, A., McGarvey, B., Brandle, J., Ma, S., Jevnikar, A., Kohalmi, S. E., and Menassa, R. (2013) High-level production of human interleukin-10 fusions in tobacco cell suspension cultures. *Plant biotechnology journal 11*, 535-45.
(44) Floss, D. M., Sack, M., Arcalis, E., Stadlmann, J., Quendler, H., Rademacher, T., Stoger, E., Scheller, J., Fischer, R., and Conrad, U. (2009) Influence of elastin-like peptide fusions on the quantity and quality of a tobacco-derived human immunodeficiency virus-neutralizing antibody. *Plant biotechnology journal 7*, 899-913.
(45) Sarangthem, V., Cho, E. A., Bae, S. M., Singh, T. D., Kim, S. J., Kim, S., Jeon, W. B., Lee, B. H., and Park, R. W. (2013) Construction and application of elastin like polypeptide containing IL-4 receptor targeting peptide. *PloS one 8*, e81891.
(46) Wang, W., Jashnani, A., Aluri, S. R., Gustafson, J. A., Hsueh, P. Y., Yarber, F., McKown, R. L., Laurie, G. W., Hamm-Alvarez, S. F., and MacKay, J. A. (2015) A thermo-responsive protein treatment for dry eyes. *J Control Release 199*, 156-67.
(47) Bidwell, G. L., 3rd, and Raucher, D. (2005) Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy. *Molecular cancer therapeutics 4*, 1076-85.
(48) Bidwell, G. L., 3rd, Davis, A. N., and Raucher, D. (2009) Targeting a c-Myc inhibitory polypeptide to specific intracellular compartments using cell penetrating peptides. *J Control Release 135*, 2-10.
(49) Bidwell, G. L., 3rd, Perkins, E., Hughes, J., Khan, M., James, J. R., and Raucher, D. (2013) Thermally targeted delivery of a c-Myc inhibitory polypeptide inhibits tumor progression and extends survival in a rat glioma model. *PloS one 8*, e55104.
(50) Ryu, J. S., and Raucher, D. (2014) Elastin-like polypeptides: the influence of its molecular weight on local hyperthermia-induced tumor accumulation. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V 88*, 382-9.
(51) Massodi, I., Thomas, E., and Raucher, D. (2009) Application of thermally responsive elastin-like polypeptide fused to a lactoferrin-derived peptide for treatment of pancreatic cancer. *Molecules 14*, 1999-2015.
(52) Massodi, I., Moktan, S., Rawat, A., Bidwell, G. L., 3rd, and Raucher, D. (2010) Inhibition of ovarian cancer cell proliferation by a cell cycle inhibitory peptide fused to a thermally responsive polypeptide carrier. *International journal of cancer. Journal international du cancer 126*, 533-44.
(53) Ryu, J. S., and Raucher, D. (2014) Anti-tumor efficacy of a therapeutic peptide based on thermo-responsive elastin-like polypeptide in combination with gemcitabine. *Cancer letters 348*, 177-84.
(54) Pinedo-Martín, G., Castro, E., Martín, L., Alonso, M., and Rodríguez-Cabello, J. C. (2014) Effect of Surfactants on the Self-Assembly of a Model Elastin-like Block Corecombinamer: From Micelles to an Aqueous Two-Phase System. *Langmuir 30*, 3432-3440.
(55) Martin, L., Castro, E., Ribeiro, A., Alonso, M., and Rodriguez-Cabello, J. C. (2012) Temperature-triggered self-assembly of elastin-like block co-recombinamers:the controlled formation of micelles and vesicles in an aqueous medium. *Biomacromolecules 13*, 293-8.
(56) Wright, E. R., and Conticello, V. P. (2002) Self-assembly of block copolymers derived from elastin-mimetic polypeptide sequences. *Adv Drug Deliv Rev 54*, 1057-73.
(57) Rodríguez-Cabello, J. C., Martín, L., Alonso, M., Arias, F. J., and Testera, A. M. (2009) "Recombinamers" as advanced materials for the post-oil age. *Polymer 50*, 5159-5169.
(58) Nettles, D. L., Chilkoti, A., and Setton, L. A. (2010) Applications of elastin-like polypeptides in tissue engineering. *Advanced Drug Delivery Reviews 62*, 1479-1485.

(59) Dreher, M. R., Simnick, A. J., Fischer, K., Smith, R. J., Patel, A., Schmidt, M., and Chilkoti, A. (2008) Temperature triggered self-assembly of polypeptides into multivalent spherical micelles. *J Am Chem Soc 130*, 687-94.
(60) Shi, P., Aluri, S., Lin, Y. A., Shah, M., Edman, M., Dhandhukia, J., Cui, H., and MacKay, J. A. (2013) Elastin-based protein polymer nanoparticles carrying drug at both corona and core suppress tumor growth in vivo. *J Control Release 171*, 330-8.
(61) Sun, G., Hsueh, P. Y., Janib, S. M., Hamm-Alvarez, S., and MacKay, J. A. (2011) Design and cellular internalization of genetically engineered polypeptide nanoparticles displaying adenovirus knob domain. *J Control Release 155*, 218-26.
(62) Na, K., Jung, J., Lee, J., and Hyun, J. (2010) Thermoresponsive pore structure of biopolymer microspheres for a smart drug carrier. *Langmuir 26*, 11165-9.
(63) Cheng, J., Park, M., Lim, D., and Hyun, J. (2013) Polypeptide microgel capsules as drug carriers. *Macromol. Res. 21*, 1163-1166.
(64) Costa, R. R., Castro, E., Arias, F. J., Rodríguez-Cabello, J. C., and Mano, J. F. (2013) Multifunctional Compartmentalized Capsules with a Hierarchical Organization from the Nano to the Macro Scales. *Biomacromolecules 14*, 2403-2410.
(65) Costa, R. R., Girotti, A., Santos, M., Arias, F. J., Mano, J. F., and Rodriguez-Cabello, J. C. (2014) Cellular uptake of multilayered capsules produced with natural and genetically engineered biomimetic macromolecules. *Acta Biomater 10*, 2653-62.
(66) McDaniel, J. R., Bhattacharyya, J., Vargo, K. B., Hassouneh, W., Hammer, D. A., and Chilkoti, A. (2013) Self-Assembly of Thermally Responsive Nanoparticles of a Genetically Encoded Peptide Polymer by Drug Conjugation. *Angewandte Chemie International Edition 52*, 1683-1687.
(67) MacKay, J. A., Chen, M., McDaniel, J. R., Liu, W., Simnick, A. J., and Chilkoti, A. (2009) Self-assembling chimeric polypeptide-doxorubicin conjugate nanoparticles that abolish tumours after a single injection. *Nat Mater 8*, 993-9.
(68) Chen, T.-H., Bae, Y., and Furgeson, D. (2008) Intelligent Biosynthetic Nanobiomaterials (IBNs) for Hyperthermic Gene Delivery. *Pharm Res 25*, 683-691.
(69) Pina, M. J., Alex, S. M., Arias, F. J., Santos, M., Rodriguez-Cabello, J. C., Mannemcherril, R. R., and Sharma, C. P. (2015) Elastin-like recombinamers with acquired functionalities for gene-delivery applications. *Journal of biomedical materials research. Part A*.
(70) Koria, P., Yagi, H., Kitagawa, Y., Megeed, Z., Nahmias, Y., Sheridan, R., and Yarmush, M. L. (2011) Self-assembling elastin-like peptides growth factor chimeric nanoparticles for the treatment of chronic wounds. *Proc Natl Acad Sci U S A 108*, 1034-9.
(71) Bessa, P. C., Machado, R., Nürnberger, S., Dopler, D., Banerjee, A., Cunha, A. M., Rodríguez-Cabello, J. C., Redl, H., van Griensven, M., Reis, R. L., and Casal, M. (2010) Thermoresponsive self-assembled elastin-based nanoparticles for delivery of BMPs. *Journal of Controlled Release 142*, 312-318.
(72) Garcia-Arevalo, C., Bermejo-Martin, J. F., Rico, L., Iglesias, V., Martin, L., Rodriguez-Cabello, J. C., and Arias, F. J. (2013) Immunomodulatory nanoparticles from elastin-like recombinamers: single-molecules for tuberculosis vaccine development. *Mol Pharm 10*, 586-97.
(73) Falk, M. H., and Issels, R. D. (2001) Hyperthermia in oncology. *Int J Hyperthermia 17*, 1-18.
(74) Simnick, A. J., Valencia, C. A., Liu, R., and Chilkoti, A. (2010) Morphing low-affinity ligands into high-avidity nanoparticles by thermally triggered self-assembly of a genetically encoded polymer. *ACS Nano 4*, 2217-27.
(75) Hassouneh, W., Fischer, K., MacEwan, S. R., Branscheid, R., Fu, C. L., Liu, R., Schmidt, M., and Chilkoti, A. (2012) Unexpected multivalent display of proteins by

temperature triggered self-assembly of elastin-like polypeptide block copolymers. *Biomacromolecules 13*, 1598-605.
(76) MacEwan, S. R., and Chilkoti, A. (2014) Controlled apoptosis by a thermally toggled nanoscale amplifier of cellular uptake. *Nano Lett 14*, 2058-64.
(77) MacEwan, S. R., and Chilkoti, A. (2012) Digital Switching of Local Arginine Density in a Genetically Encoded Self-Assembled Polypeptide Nanoparticle Controls Cellular Uptake. *Nano Letters 12*, 3322-3328.
(78) Liu, W., MacKay, J. A., Dreher, M. R., Chen, M., McDaniel, J. R., Simnick, A. J., Callahan, D. J., Zalutsky, M. R., and Chilkoti, A. (2010) Injectable intratumoral depot of thermally responsive polypeptide-radionuclide conjugates delays tumor progression in a mouse model. *J Control Release 144*, 2-9.
(79) Hoare, T. R., and Kohane, D. S. (2008) Hydrogels in drug delivery: Progress and challenges. *Polymer 49*, 1993-2007.
(80) Altunbas, A., and Pochan, D. (2012) Peptide-Based and Polypeptide-Based Hydrogels for Drug Delivery and Tissue Engineering, in *Peptide-Based Materials* (Deming, T., Ed.) pp 135-167, Springer Berlin Heidelberg.
(81) Yan, C., and Pochan, D. J. (2010) Rheological properties of peptide-based hydrogels for biomedical and other applications. *Chemical Society Reviews 39*, 3528-3540.
(82) Evans, C. H., Kraus, V. B., and Setton, L. A. (2014) Progress in intra-articular therapy. *Nat Rev Rheumatol 10*, 11-22.
(83) Floss, D. M., Schallau, K., Rose-John, S., Conrad, U., and Scheller, J. (2010) Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. *Trends in Biotechnology 28*, 37-45.
(84) Hassouneh, W., MacEwan, S. R., and Chilkoti, A. (2012) Fusions of elastin-like polypeptides to pharmaceutical proteins. *Methods Enzymol 502*, 215-37.
(85) Meyer, D. E., and Chilkoti, A. (2002) Protein purification by inverse transition cycling in *Protein Interactions E Golemis, Ed. Cold Spring Harbor Laboratory Press* (Golemis, E., and Adams, P. D., Eds.) pp 938, Cold Spring Harbor Laboratory Press.
(86) Amiram, M., Luginbuhl, K. M., Li, X., Feinglos, M. N., and Chilkoti, A. (2013) A depot-forming glucagon-like peptide-1 fusion protein reduces blood glucose for five days with a single injection. *J Control Release 172*, 144-51.
(87) Amiram, M., Luginbuhl, K. M., Li, X., Feinglos, M. N., and Chilkoti, A. (2013) Injectable protease-operated depots of glucagon-like peptide-1 provide extended and tunable glucose control. *Proc Natl Acad Sci U S A 110*, 2792-7.
(88) Betre, H., Liu, W., Zalutsky, M. R., Chilkoti, A., Kraus, V. B., and Setton, L. A. (2006) A thermally responsive biopolymer for intra-articular drug delivery. *J Control Release 115*, 175-82.
(89) Shamji, M. F., Betre, H., Kraus, V. B., Chen, J., Chilkoti, A., Pichika, R., Masuda, K., and Setton, L. A. (2007) Development and characterization of a fusion protein between thermally responsive elastin-like polypeptide and interleukin-1 receptor antagonist: sustained release of a local antiinflammatory therapeutic. *Arthritis Rheum 56*, 3650-61.
(90) Shamji, M. F., Whitlatch, L., Friedman, A. H., Richardson, W. J., Chilkoti, A., and Setton, L. A. (2008) An injectable and in situ-gelling biopolymer for sustained drug release following perineural administration. *Spine (Phila Pa 1976) 33*, 748-54.
(91) Sinclair, S. M., Bhattacharyya, J., McDaniel, J. R., Gooden, D. M., Gopalaswamy, R., Chilkoti, A., and Setton, L. A. (2013) A genetically engineered thermally responsive sustained release curcumin depot to treat neuroinflammation. *J Control Release 171*, 38-47.

(92) Liu, W., McDaniel, J., Li, X., Asai, D., Quiroz, F. G., Schaal, J., Park, J. S., Zalutsky, M., and Chilkoti, A. (2012) Brachytherapy using injectable seeds that are self-assembled from genetically encoded polypeptides in situ. *Cancer research 72*, 5956-65.
(93) Asai, D., Xu, D., Liu, W., Garcia Quiroz, F., Callahan, D. J., Zalutsky, M. R., Craig, S. L., and Chilkoti, A. (2012) Protein polymer hydrogels by in situ, rapid and reversible self-gelation. *Biomaterials 33*, 5451-8.
(94) Fernandez-Colino, A., Arias, F. J., Alonso, M., and Rodriguez-Cabello, J. C. (2014) Self-organized ECM-mimetic model based on an amphiphilic multiblock silk-elastin-like corecombinamer with a concomitant dual physical gelation process. *Biomacromolecules 15*, 3781-93.
(95) Xia, X. X., Xu, Q., Hu, X., Qin, G., and Kaplan, D. L. (2011) Tunable self-assembly of genetically engineered silk--elastin-like protein polymers. *Biomacromolecules 12*, 3844-50.
(96) Gustafson, J. A., Price, R. A., Frandsen, J., Henak, C. R., Cappello, J., and Ghandehari, H. (2013) Synthesis and characterization of a matrix-metalloproteinase responsive silk-elastinlike protein polymer. *Biomacromolecules 14*, 618-25.
(97) Hu, X., Wang, X., Rnjak, J., Weiss, A. S., and Kaplan, D. L. (2010) Biomaterials derived from silk-tropoelastin protein systems. *Biomaterials 31*, 8121-31.
(98) Cappello, J., Crissman, J. W., Crissman, M., Ferrari, F. A., Textor, G., Wallis, O., Whitledge, J. R., Zhou, X., Burman, D., Aukerman, L., and Stedronsky, E. R. (1998) In-situ self-assembling protein polymer gel systems for administration, delivery, and release of drugs. *J Control Release 53*, 105-17.
(99) Megeed, Z., Cappello, J., and Ghandehari, H. (2002) Genetically engineered silk-elastinlike protein polymers for controlled drug delivery. *Adv. Drug Delivery Rev. 54*, 1075-1091.
(100) Huang, W., Rollett, A., and Kaplan, D. L. (2014) Silk-elastin-like protein biomaterials for the controlled delivery of therapeutics. *Expert Opinion on Drug Delivery*, 1-13.
(101) Yucel, T., Lovett, M. L., and Kaplan, D. L. (2014) Silk-based biomaterials for sustained drug delivery. *J Control Release 190*, 381-97.
(102) Greish, K., Frandsen, J., Scharff, S., Gustafson, J., Cappello, J., Li, D., O'Malley, B. W., Jr., and Ghandehari, H. (2010) Silk-elastinlike protein polymers improve the efficacy of adenovirus thymidine kinase enzyme prodrug therapy of head and neck tumors. *J Gene Med 12*, 572-9.
(103) Gustafson, J. A., Price, R. A., Frandsen, J., Henak, C. R., Cappello, J., and Ghandehari, H. (2013) Synthesis and Characterization of a Matrix-Metalloproteinase Responsive Silk–Elastinlike Protein Polymer. *Biomacromolecules 14*, 618-625.
(104) Hennink, W. E., and van Nostrum, C. F. (2002) Novel crosslinking methods to design hydrogels. *Advanced Drug Delivery Reviews 54*, 13-36.
(105) Testera, A. M., Girotti, A., Torre, I. G. d., Quintanilla, L., Santos, M., Alonso, M., and Rodríguez-Cabello, J. C. (2015) Biocompatible elastin-like click gels: design, synthesis and characterization. *Journal of Materials Science: Materials in Medicine*
(106) Gonzalez de Torre, I., Santos, M., Quintanilla, L., Testera, A., Alonso, M., and Rodriguez Cabello, J. C. (2014) Elastin-like recombinamer catalyst-free click gels: characterization of poroelastic and intrinsic viscoelastic properties. *Acta Biomater 10*, 2495-505.
(107) de Torre, I. G., Wolf, F., Santos, M., Rongen, L., Alonso, M., Jockenhoevel, S., Rodriguez-Cabello, J. C., and Mela, P. (2015) Elastin-like recombinamer-covered stents: Towards a fully biocompatible and non-thrombogenic device for cardiovascular diseases. *Acta Biomater 12*, 146-55.

(108) Hu, B.-H., and Messersmith, P. B. (2003) Rational Design of Transglutaminase Substrate Peptides for Rapid Enzymatic Formation of Hydrogels. *Journal of the American Chemical Society 125*, 14298-14299.
(109) Bandiera, A., Markulin, A., Corich, L., Vita, F., and Borelli, V. (2014) Stimuli-induced release of compounds from elastin biomimetic matrix. *Biomacromolecules 15*, 416-22.