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# Title: Elastin-like recombinamers as smart drug delivery systems

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**Abstract:** Drug delivery systems that are able to control the release of bioactive molecules and designed to carry drugs to target sites are of particular interest for tissue therapy. Moreover, systems comprising materials that can respond to environmental stimuli and promote self-assembly and higher order supramolecular organization are especially useful in the biomedical field. Suitable biomaterials for this purpose include elastin-like recombinamers (ELRs), a class of proteinaceous polymers bioinspired by natural elastin and designed using recombinant technologies. The self-assembly and thermoresponsive behaviour of these systems, along with their biodegradability, biocompatibility and well-defined composition as a result of their tailor-made design, make them particularly attractive for drug delivery.

This review brings together information concerning ELR-based delivery systems that allow targeted delivery, especially ELR-drug recombinant fusion constructs, ELR-drug systems chemically bioconjugated in their monomeric and soluble forms, and drug encapsulation by nanoparticle-forming ELRs. Subsequently, we will focus on those drug carriers in which smart release is triggered by pH or temperature with a particular focus on cancer treatments in which both enhanced permeability retention (EPR) and local mild hyperthermia cause ELRs to become aggregated or form well-defined nanoparticles at the site of action. Systems for controlled drug release based on depots and hydrogels that act as both a support and reservoir in which drugs can be stored will be described, and their applications in drug delivery discussed. Finally, smart drug-delivery systems not based on ELRs, including those comprising proteins, synthetic polymers and non-polymeric systems, will also be briefly discussed.

**Keywords:** Elastin-like Recombinamers, Drug delivery, Smart systems, Self-Assembly, Hydrogels, Nanocarriers.

## 1. INTRODUCTION

The next generation of biomaterials will require new components capable of undergoing bioresponsive self-assembly and higher order supramolecular organization. In this sense, polymers that show lower critical solution temperature (LCST) phase-transition behaviour are a class of materials that can be used as fundamental pillars which are capable of interacting with biological systems at the molecular level [1].

A great variety of drug-delivery systems have been developed to obtain more efficient treatments and to deal with the largest possible number of diseases [2]. Recently, one of the main goals has been to develop systems that are capable of exhibiting a responsive behaviour to different environmental changes. Both polymeric and non-polymeric structures have been included in the wide variety of drug-delivery systems designed, including micelles, liposomes, polymer-drug conjugates or inorganic nanoparticles, each with different benefits [3, 4].

Advanced drug-delivery systems (DDSs) have been designed to transport pharmaceuticals to target tissues and to control drug release by responding to certain stimuli. Thus, DDSs will improve therapeutic effects, reduce drug toxicity and improve the release profile with respect to the administration of free drugs, and in some cases can even penetrate into the targeted cells if required. Stimuli-responsive systems have become increasingly important in drug delivery due to their ability to rapidly respond to specific stimuli or to changes in their environment, including pH [5], temperature, ion concentration [6], light [7] and other changes [8]. Different materials, including natural or synthetic polymers, can be used to design such stimuli-responsive systems. Natural polymers such as proteins also exhibit several characteristic advantages when used as a biomaterial to form drug carriers as they are able to form nanostructures according to their established secondary, tertiary and quaternary structures. Secondary structures comprise those such as an  $\alpha$ -helix or  $\beta$ -sheet that can be used to bind micro- and nanostructures together [9, 10]. They have a versatile structure because of the broad range of design possibilities conferred by the high number of possible combinations of amino acids. In addition, proteins are made by living organisms and therefore have a higher likelihood of resembling natural tissues and being degraded by physiological pathways into the corresponding non-toxic byproducts. However, natural proteins vary from one batch to the next and can contain impurities, contaminants and infectious agents or cause hypersensitivity

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reactions. Genetic engineering has overcome these disadvantages and has allowed the creation and improvement of new bioinspired protein-based polymers with enhanced structural and functional features with respect to their natural analogues. As such, these polymers are excellent candidates for the design of drug-delivery systems. Examples of genetically engineered well-developed drug carriers include protein polymers composed from elastin-like polypeptides due to their thermoresponsive behaviour and ability to coacervate.

Elastin-like recombinamers (ELRs) are synthetic proteinaceous polymers bioinspired by natural elastin sequences and obtained using recombinant DNA techniques, thus allowing strict control of their amino acid composition and subsequent properties [11]. ELR genes are commonly expressed in *Escherichia coli* and purified by several heating and cooling cycles followed by centrifugation, in a process known as inverse temperature cycling (ITC).

ELRs are based on the recurrence of short repeating peptide motifs present in tropoelastin; one of the most common repetitions is the pentapeptide sequence (Val-Pro-Gly-Xaa-Gly), where Xaa is any amino acid except L-Proline. This type of polymer has a wide range of interesting properties, most of them derived from natural elastin, such as their biocompatibility, excellent mechanical properties, ability to self-assemble [12] and stimuli-responsive behaviour [13]. Moreover, these polymers are able to respond to different environmental stimuli, such as pH, ion concentration, pressure or temperature [8], with the latter being the most widely studied variable. The transition temperature ( $T_t$ ) is the characteristic temperature below which the polymer chains remain hydrated, disordered and relatively extended, and above which the ELR chain folds hydrophobically and assembles to form a separate phase. In this state the chains adopt a dynamic secondary structure known as a  $\beta$ -spiral [12, 14]. This  $T_t$  is controlled by intrinsic parameters, including composition, molecular weight and hydrophobicity, and it is also influenced by extrinsic factors such as concentration, solutes or pH. The tailor-made design of ELRs enables their transition temperature to be tuned and adapted for specific applications.

ELRs have been used in a wide variety of applications in various biomedical fields such as drug delivery [15], tissue engineering [16], regenerative medicine [17, 18] and other medical devices [19]. The purpose of this review is to collect information about ELR-based delivery systems that promote target delivery, subsequently focusing on those drug carriers in which release is triggered by pH or temperature stimuli, especially depots and hydrogels that act as drug reservoirs and others not based on ELRs that have recently found uses as smart drug-delivery systems.

Three different types of ELR constructs can be distinguished based on the different strategies used to design targeted drug-delivery systems. Firstly, ELR-drug recombinant fusion constructs, in which therapeutic sequences are fused to the backbone of the ELR in what is known as ELPylation. This system allows both expression and purification to be enhanced while improving drug delivery and pharmacokinetics [20]. The second type of targeted drug-delivering ELRs consist of chemically bioconjugated

recombinamers. In this case, modification is achieved by introduction of a “cross-linking” site in amino acid residues of the ELR in order to fuse a drug, thus allowing it to be delivered at the appropriate site and improving its pharmacokinetic properties (3). The final strategy described is based on drug encapsulation by ELR-based nanoparticles. A wide range of encapsulating ELRs have been studied, among which block copolymers that can form micelles by self-assembly in response to different parameters (27, 33A), chemically cross-linked microgel capsules and other systems used in gene delivery (37) can be highlighted.

With respect to drug delivery triggered by temperature, the thermosensitive characteristics of ELRs make them one of the most interesting systems as regards a response to pathological changes in temperature. Hyperthermia, or local heating of the tumour site, is an excellent stimulus for the development of new anti-cancer therapies as a result of the vasodilatation [21], vascular permeability [22] and increased intracellular traffic into the tumour cells [23]. These effects may allow successful drug delivery by ELR-based systems as they are thermoresponsive and capable of changing from a soluble state to an insoluble structure with the formation of aggregates or self-assembly into well-defined nanoparticles at the disease site. Thus, these smart architecture features have been applied in the biomedical field specifically for drug-delivery purposes. Very little research has been devoted to the development of smart pH-sensitive ELRs, with recent proposals being related to ELR-based micelles with an ability to self-assemble and with pH-responsiveness [24] and pH-mediated drug delivery based on ELRs with an acid-labile linker [25].

One of the suggestions for controlling the release of therapeutic agents and avoiding a fluctuating saw-tooth-like release is to apply a depot or hydrogel system that acts as a support and reservoir in which the drug can be stored [26]. The role of this system is to allow controlled delivery of the drug that can be tuned as a result of the different features derived from material design as well as polymer and drug concentrations, inter-solubility or matrix degradation simultaneous with drug release.

Depot formation is governed by the smart behaviour and temperature sensitivity of ELRs; this, along with a tailor-made structure designed to possess a  $T_t$  below the physiological temperature, makes them injectable systems. Thus, the ELR solution is injected at the site of action as a soluble ELR (unimers), and the polymers spontaneously coacervate to form the “drug depot” as a consequence of the temperature. In particular, regenerative medicine, neuroinflammation and cancer and diabetes therapy have benefited from this thermoresponsive behaviour [27]. Depending on the designed structure of the ELRs, a more organized polymeric network with a 3D structure can be obtained in hydrogels in which interchain connections can be provided by physical or chemical linkages. The interconnected 3D structure of the hydrogel, together with its ability to contain a large amount of water inside, provides a friendly environment for encapsulating therapeutic agents. Indeed, injectable hydrogels formed by *in situ* polymer crosslinking are of increasing interest for drug delivery as they enable a depot to be directly formed *in vivo* in the target tissue with just an injection and avoiding surgical

implantation [28-30]. Thus, direct application of the ELR at the target site helps to extend the local drug exposure time while minimizing systemic side effects [31]. Depending on the complexity of the ELR structure and the nature of the crosslinking, several different physical and chemical hydrogels will be described highlighting their different applications resulting from the specific bioactive molecule released.

Finally, an overview of drug-delivery systems other than those based on ELRs will be provided. There are a wide variety of structures for drug-delivery systems made up of both polymeric and non-polymeric building blocks [32]. Some examples of natural proteins used in delivery systems, such as silk and collagen, will be described together with non-polymeric carriers defined by non-repetitive amino acid motifs with an ability to acquire tertiary or quaternary structures, including vault proteins [33] and viral proteins [34]. Subsequently, and staying with synthetic polymers, applications of poly(lactic-co-glycolic acid) (PLGA) in drug delivery will be reviewed [35], finishing with non-polymeric systems such as metal organic frameworks (MOFs) and their applications.

## 2. TARGETED DRUG DELIVERY MEDIATED BY ELASTIN-LIKE RECOMBINAMERS

Elastin-like recombinamers (ELRs) have been widely engineered to be suitable for application in targeted drug delivery without the need for any type of sensitivity to ensure drug release. Three different classes can be established based on the different strategies designed for this purpose, namely ELR-drug recombinant fusion constructs, ELR-drug combinations chemically bioconjugated in their monomeric and soluble form, and drug encapsulation by nanoparticle-forming ELRs.

### 2.1. ELR-drug fusion constructs

Recombinant DNA techniques have enormous potential for the construction of therapeutic proteins fused to an ELR backbone, which is often called ELPylation (for elastin-like polypeptide) [20]. This approach allows us to take advantage of a purification method that relies on the transition temperature 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). This system permits expression and purification to be enhanced while improving drug delivery and pharmacokinetics [36].

With this premise, an ELR was engineered fused to a poly-aspartic acid tail (named ELR-D) with the aim of achieving self-assembled nanoparticles whose size is regulated by the charge repulsion of the aspartic acid tails. In order to add a biofunctionality to the nanoparticles formed, EGF (epidermal growth factor) was fused to the construct (to form the so-called ELR-D-EGF), being displayed on the surface of ELR-D. The binding of EGF to its receptor on the cell surface was assessed upon culture with A549 cells, a human lung adenocarcinoma epithelial cell line that overexpresses the EGF receptor, thereby confirming the expected increase in cell proliferation upon EGF binding when compared to the non-EGF-fused control. This system was also shown to be a good candidate for drug delivery applications by the loading

of paclitaxel, a well-known anti-cancer drug, which showed a better internalization into A549 cells inducing cell death [37].

In another study, a novel strategy involving fusion of a single-chain vascular endothelial growth factor (VEGF<sub>121</sub>)-derived polypeptide to an elastin-like recombinamer was designed with the aim of enhancing the purification and *in vivo* stability of growth factors. This method was denominated as purification and delivery system (PADS) and the fusion product as VEGF-PADS. The ELR-VEGF protein was successfully purified by ITC and evaluated *in vitro* in human umbilical vein cell (HUVEC) culture by means of vascular tube formation and cell proliferation and migration, showing results significantly comparable to free VEGF, thereby confirming the bioactive properties of the chimera and suggesting that the ELR tag has no effect on the binding of VEGF<sub>121</sub> to its receptor. This approach also led to slower plasma clearance of the growth factor when injected in mice (half the rate for free VEGF<sub>121</sub>), although biodistribution was different for each one, with the former mainly being located in the liver and the free growth factor in the kidney. This work shows the potential of fusing growth factors to ELRs in order to lower the purification cost while maintaining their bioactivity [38].

Likewise, taking advantage of the ability of some ELRs to aggregate into coacervates when the temperature is raised above the transition temperature, a keratinocyte growth factor (KGF)-ELR chimera was recombinantly designed and bioproduced. Subsequent use of this material *in vivo* for the treatment of chronic wounds in diabetic mice led to enhancement of the granulation and re-epithelization of the wound when compared with the effect of KGF entrapped in fibrin gels due to the sustained administration of KGF in the ELR coacervate [39].

In order to improve the treatment of dry eyes, lacritin (Lactr), a protein component of human tears with pro-secretory activity in the lacrimal gland, was fused to an ELR comprising the VPGVG pentapeptide, thus allowing localization of the drug at the site of action due to precipitation of the recombinamer at physiological temperature, above the T<sub>t</sub>, and overcoming the limitation of rapid tear turnover of single Lactr when tested *in vivo* [40]. Another approach involving this same system combined the ability of a block copolymer (see following section concerning encapsulation systems) consisting of hydrophilic (serine-containing) and hydrophobic (isoleucine-containing) elastin-derived domains, thus being able to form nanoparticles at 37 °C, with the therapeutic properties of lacritin, which led to a faster regeneration of the corneal epithelium after wound induction in non-obese diabetic (NOD) mice when compared to an ELR-Lactr that does not assemble into particles [41]. Similarly, the knob domain of the fibre capsid protein of adenovirus serotype 5, previously bound to an ELR and confirmed to improve internalization into hepatocytes [42], which was shown to bind to the coxsackievirus and adenovirus receptor highly expressed in the lacrimal gland, was fused to the latter ELR-Lactr nanoparticle, thus allowing selective transcytosis of the ELR-drug fusion into the cells of this gland [43].

The recombinant fusion of anti-inflammatory proteins, such as antibodies that recognize pro-inflammatory cytokines, has also been achieved using the above-mentioned ELPylation approach. Due to the limitations of prokaryotic recombinant expression systems regarding post-translational modifications that most of these anti-inflammatory proteins undergo, many of them have been expressed in plants. Some of the first studies performed in this bioproduction system were aimed at evaluating the expression and purification of different proteins fused to ELRs in tobacco leaves, with satisfactory results [44, 45]. With regard to antibodies, anti-human TNF was also fused to an ELR, produced in *Nicotiana tabacum* and purified by ITC. Further investigation was performed both *in vitro* and *in vivo* and showed both similar bioactivity to free TNF and an increase from 28 minutes to 11 hours (24-fold) in terms of serum half-life and clearance, thus indicating an improvement in the pharmacokinetics of the antibody [46]. Similarly, IL-10 [47] and an HIV-neutralizing antibody [48] were also engineered into an ELR-bound DNA vector for expression in tobacco plants, showing analogous bioactivity to that of the free proteins without the need for cleavage from the ELR tag. Nevertheless, the use of peptides instead of whole proteins for fusion to ELRs allows the expression in more common systems such as *E. coli*, thereby reducing costs and usually performing better in terms of yields. As an example, six repetitions of the peptide AP1, a ligand for IL-4 receptors (IL-4Rs), were genetically bound to a VPGVG-based ELR. The resulting chimeric recombinamer was fluorescently labelled and found to bind to IL-4Rs *in vitro* in different tumour cell lines in which IL-4Rs are highly expressed, thus leading to an amplification of the expression of some anti-apoptotic proteins and preventing drug-induced cell death. The system tested also resulted in tumour accumulation *in vivo*, with this effect being evident as early as 10 minutes post-injection of the ELR-AP1, peaking at 6 h and being maintained up to 24 h [49]. This carrier should therefore be tested as an actual drug-delivery system with a chemotherapeutic agent.

Alternatively, self-assembled ELRs have been designed as nanovaccines against tuberculosis by the recombinant fusion of an antigen from *Mycobacterium tuberculosis*. In this case, a nanoparticle-forming di-block consisting of a glutamic acid (E) hydrophilic block and an isoleucine (I) hydrophobic one, with different amino acids as guest residues, was chosen. The resulting particles were found to be monodisperse and stable despite fusion to the antigen, and additional work was performed in order to assess the ability of the nanovaccine to elicit an immune response *in vivo*, thus resulting in the triggering of an innate response following by the presence of IL-5 and IgM and IgG, which clearly suggests an adaptive Th2 response. Meanwhile, the ELR itself did not have any immunomodulatory effect. In conclusion, these results suggest the potential application of ELRs as antigen carriers [50].

## 2.2. Chemically bioconjugated ELR-drug constructs

Covalent bioconjugation has been extensively used to achieve therapeutic elastin-like recombinamers chemically fused to drugs, thus allowing an improvement in the solubility of hydrophobic drugs and in the pharmacokinetics of these drugs. This covalent binding is performed by

introducing cross-linking sites via the modification of reactive groups in the side chains of different amino acids, such as thiol groups in cysteine or the  $\epsilon$ -amino group in lysine residues. In contrast to the recombinant fusion, this approach implies that non-protein drugs can be bioconjugated.

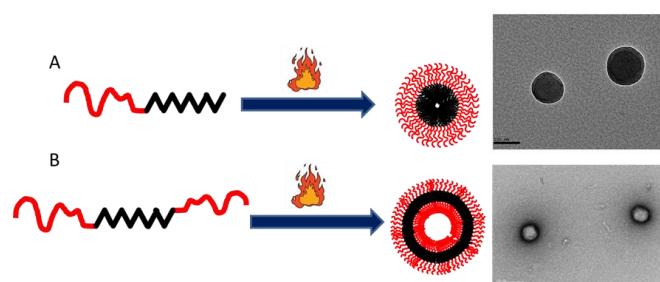
Doxorubicin (Dox), a widely used chemotherapeutic agent, has been bioconjugated to several ELRs. One of the first studies in this regard accomplished such conjugation by modifying the amine groups present in lysine residues of the ELR to obtain a free maleimide group, which reacted with a Dox-hydrazone complex containing an acid-labile hydrazine bond to achieve bioconjugation. Further *in vitro* studies performed to assess the cytotoxicity and subcellular localization of the ELR-Dox showed a similar level of squamous cell carcinoma (FaDu) cell death (less than 10% of survival after 72 h) when compared to the free drug, although different localizations were observed, with ELR-Dox being dispersed throughout the cytoplasm and free doxorubicin mostly internalized in the nucleus, suggesting different cell-death pathways in each case [51]. Another possibility involving chemical conjugation via cysteine residues was evaluated and an ELR-Dox drug system synthesized, this system allowing intracellular release of the drug as a result of an acid-labile hydrazine bond [52]. An alternative approach using the latter recombinamer involved the inclusion of a GFLG tetrapeptide linker that is recognized by lysosomal cathepsin proteases, thus permitting the release of the drug after endocytosis. A Tat domain, which is a cell-penetrating peptide (CPP) derived from the trans-activating protein found in HIV-1, was also fused to this recombinamer, theoretically improving cellular uptake of the conjugated drug. *In vitro* studies were performed with both non- and Dox-resistant carcinoma cell lines, resulting in a higher cytotoxicity towards drug-resistant cells in the case of the ELR-Dox when compared to the drug itself (1.4% and 31.9% resistance, respectively, in the case of breast cancer cells) [53]. Additionally, a nanoparticle-forming block copolymer ELR was used to conjugate doxorubicin via cysteine residues. Hence, 40nm particles were achieved above the Tt of the ELR, with little effect of Dox on this feature and the drug being located at the core of the nanoparticle. Tumour regression was observed in *in vivo* assays using a mouse colon cancer model after a single dose, which represents a great advantage with respect to the free drug [54]. Furthermore, other chemotherapeutic drugs, such as paclitaxel, have been bioconjugated and found to present the same benefits as Dox in terms of pharmacokinetics and improved solubility [55].

As an example of a likely ELR as a drug carrier for maternal delivery and prevention of foetal exposure, an ELR-fluorescent probe system was designed in order to demonstrate the potential of this class of polypeptides for use in such applications, starting from the hypothesis of the lower ability of these proteins to cross the placental barrier compared to free drugs. The covalent binding of a fluorescent dye to an ELR or a SynB1-ELR backbone, with SynB1 being another CPP, allowed traceability when injected intravenously into a pregnant rat model. The *ex vivo* fluorescent measurement of the placenta and the foetus showed that very small quantities of the labelled ELR crossed the placenta, whereas the observation of different

maternal organs showed a preferential accumulation of the ELR in kidneys, liver and the placenta itself, thereby suggesting the potential of elastin-like recombinamers as safe drug carriers for different diseases, such as preeclampsia [56].

### 2.3. Elastin-like recombinamers as non-responsive encapsulation systems

The aforementioned recombinant nature of ELRs permits a strict control over their physical and chemical properties, thus allowing the tailoring of their self-assembly into different structures, such as different types of nanoparticles, depending on the amino acid present as guest residue in the VPGXG pentapeptide. One of the most widely explored possibilities is the construction of ELRs consisting of hydrophobic and hydrophilic blocks, known as “block copolymers”. The design of a di-block recombinamer implies that it undergoes a conformational change from an extended form to micelles above the Tt of the hydrophobic part [57, 58]. In this case, the Tt is called the critical micelle temperature (CMT) and can be controlled by varying the length and composition of the hydrophobic block, while the size of the micelle depends mostly on the length of the ELR and the hydrophilic-to-hydrophobic block ratio. The core of the micelle is suggested to be occupied by the hydrophobic block, while the hydrophilic one is oriented towards the corona, in contact with the surrounding water molecules. Further investigation regarding the self-assembly behaviour of ELRs into micelles or hollow vesicles (see ¡Error! No se encuentra el origen de la referencia.) involved changing the arrangement and length of the constituent blocks, leading to the conclusion that these two different structures can be obtained using ELRs comprising glutamic acid- and alanine-based blocks, thereby suggesting the potential functionalization of these recombinamers for subsequent applications [59]. Additionally, the effect of surfactants [60] and different salts [61] on ELR self-assembly was assessed and it was found that these conditions can completely modify the structure of the self-assembled ELRs, i.e. the formation of micelles or vesicles, depending on the concentration of these additives in the aqueous recombinamer solution.



**Figure 1.** TEM images of self-assembled nanoparticles stained with 1% uranyl acetate of the ELR enriched with glutamic and alanine. Micelle like (A) and vesicle like self-assembled ELR architecture (B) [59].

With the purpose of exploring the possibility of adding novel bioactivities to ELRs in order to enhance their drug-delivery features, *Plasmodium falciparum* FK506-binding protein (FKBP), which binds to the chemotherapeutic anti-proliferative drug rapamycin, was fused to the hydrophilic

part of a di-block micelle-forming recombinamer. This modification increased the affinity of the ELR for this drug since FKBP is exposed in the corona of the nanoparticle. *In*

*vitro* studies in a breast cancer cell line showed an increase in the loading capacity of the drug by the particles and its release, therefore improving its activity towards these tumour cells [62].

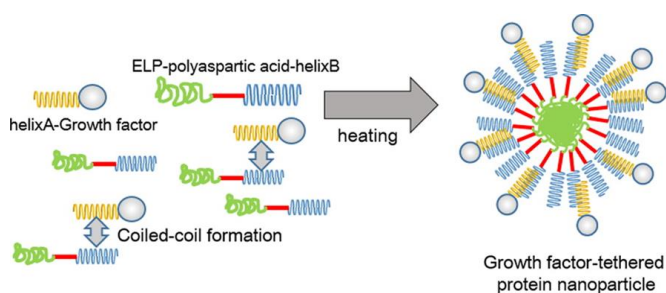
Chemical cross-linking can be used as another method for the development of thermo-responsive microgel capsules (MCs) with potential applications in drug delivery. This approach permits shape-stable microspheres, the pore size of which can be reversibly tuned by increasing or lowering the temperature, thereby modifying the permeability to drugs, to be obtained. In a first example of this technology, a mixture of ELR and albumin at different ratios was cross-linked, with the resulting system showing shape stability and changes in the surface structure of the MCs depending on whether the temperature was above or below the Tt. In the first case, the release of BSA and prednisone acetate, both used as model drugs, was faster than in the latter. Optimal release was found in the temperature range 20-40 °C [63]. ELR/BSA MCs were subsequently produced by a two-step cross-linking method. The ability of these microcapsules to release a drug was evaluated using two similar fluorophores, namely rhodamine B (red) and FITC (green), this latter possessing a negative charge, thus showing a charge-independent release of both molecules from the MCs. Similarly to the previous work, release of the cargo molecules was faster when the temperature was raised above Tt, and vice versa [64].

Dual microparticles consisting of two different biomaterials, namely ELR-chitosan or alginate-chitosan, have also been designed as a proof-of-concept methodology following a layer-by-layer approach and found to have potential as drug-delivery systems [65]. Further studies with the ELR-chitosan microparticles involved recombinant functionalization of the ELR with the RGD cell-adhesion sequence for integrins. These particles, with a size of 3-4 µm, were loaded with fluorescently labelled DQ-ovoalbumin as a model protein and used to supplement the medium in human mesenchymal stem cells (hMSCs) culture for 72 h. The findings of this study showed them to be non-cytotoxic, with improved internalization of the protein to the cytoplasm than in the case of the non-functionalized (non-RGD) ELR-chitosan microparticles, as evidenced by DQ-ovoalbumin degradation, which results in a shift in fluorescence emission. Thus, this strategy suggests the utilization of microparticles formed by different biocompatible macromolecules as carriers for drug delivery [66].

In a more recent work, elastin-like-silica hybrid micelles were obtained by fusing the silaffin R5 peptide to an ELR. Silaffins are polycationic peptides with affinity for silica found in *Cylindrotheca fusiformis*, playing a role in the formation of its silica cell walls. However, on their own these peptides self-assemble into uncontrolled structures, thus leading to a silicification process resulting in a network of fused silica nanoparticles. The fusion of this peptide to an ELR capable of self-assembling into micelles permits the synthesis of uniform hybrid ELR and silica nanoparticles that could be used as drug-delivery systems, as shown by the higher monodispersity and larger silica-coated micelles of

the silaffin-containing recombinamer when compared with the micelle-forming non-silaffin counterpart [67].

The same group that engineered the ELR-D-EGF fusion protein discussed above designed another approach for loading of the previously described single-chain vascular endothelial growth factor (scVEGF<sub>121</sub>)-derived polypeptide into nanoparticles. This approach takes advantage of the non-covalent interaction between coiled-coil leucine zipper domains, one with acid (helix A) and another one with basic (helix B) residues, both peptides being designed to form stable, parallel, coiled-coil heterodimers (**Figure 2**). An ELR-D-helixB and a helixA-scVEGF<sub>121</sub> were designed and recombinantly produced in *E. coli* and found to form coiled-coil nanoparticles. Further experiments involving the loading of paclitaxel into the nanoparticles showed the enhanced cytotoxicity of the resulting drug-delivery system upon supplementing the medium with these particles in the culture of HeLa cells. This study showed uptake of these particles by the cells and the release of paclitaxel specifically via the VEGF receptor [68].



**Figure 2.** Schematic representation of the tethering of the growth factor to nanoparticles through the formation of coiled-coil assemblies. Reprinted with permission from Assal *et al.* [68].

Other novel encapsulation systems have been designed recently taking advantage of the properties of other recombinamers resulting from combination of the VPGAVG hexapeptide, also found in human elastin, and VPGVG sequences, together with crosslinking regions that permit further modification of the ELR. This ELR was found to form microparticles ( $11 \pm 5 \mu\text{m}$ ) via a water-in-oil emulsion, which were combined with and covalently attached to superparamagnetic iron oxide nanoparticles with the aim of applying a controlled magnetic field once implanted *in vivo* and improving the targeting and tracking of these vectors when loaded with a drug. To assess the actual potential of these microparticles, they were loaded with either fluorescently labelled bovine serum albumin (BSA) as a model drug or neural growth factor (NGF) for *in vitro* release studies. In the latter study, a neural differentiation of the PC12 cell line was also observed. However, this study was quite limited and some improvements are still required, especially a reduction in particle size in order to be suitable for *in vivo* applications [69].

Various studies have also been performed with ELRs as gene-delivery systems, with all of them relying on the electrostatic interactions between negatively charged DNA molecules and positively charged ELRs due to substitution of the guest amino acid in the VPGXG pentapeptide by an amine-containing residue, such as lysine, or the fusion of a

tail including positively charged amino acids. The latter approach has been used to attach an oligolysine tail (K8) to the ELR to give the so-called K8-ELR, which was subsequently incubated with plasmid DNA (pDNA) including the gene encoding for green fluorescent protein (GFP) and found to exhibit good complexation and stability of the polyplexes. The transfection efficiency was also assessed by way of culture with *in vitro* MCF-7 breast cancer cells, with fluorescence due to GFP expression being observed. However, some cytotoxic effects were also observed, probably due to the K8 tail [70]. In another recent work, a positively charged ELR was fused to the penetratin cell-penetrating peptide and the LAEL fusogenic peptide. Stable polyplexes of around 200 nm, with a zeta-potential of +24 mV were formed and found to be optimal for the desired application. Haemocompatibility and cytotoxicity assays showed the innocuous nature of these ELRs. Furthermore, the LAEL-containing ELR was found to be the best choice in terms of cellular uptake and transfection among the ELRs tested. These results show the potential of highly biocompatible ELRs for gene-therapy applications [71].

### 3. DRUG DELIVERY TRIGGERED BY ELR SMART BEHAVIOUR

As stated above, soluble ELRs suffer a reversible phase transition in response to changes in temperature which, depending on their architecture, causes them to aggregate or self-assemble into well-defined nanoparticles. As a result, these smart architectural features have been applied in the biomedical field, especially for drug-delivery purposes.

#### 3.1. Drug delivery triggered by temperature: hyperthermia and cancer therapy

##### 3.1.1. ELR aggregates

The use of drug delivery systems for cancer treatment is dependent on the enhanced permeability and retention (EPR) effect present in tumour tissues. The EPR effect is characterized by the accumulation of macromolecules and lipids as a consequence of restricted lymphatic drainage. The regional blood flow to the tumour, the permeability of the tumour vasculature, the structural barriers imposed by perivascular tumour cells and extracellular matrix, together with intratumoural pressure, must all be taken into account in the design of novel drug-delivery agents [72]. The combination of an EPR with local mild hyperthermia (40-44 °C) [73] makes these constructs appropriate candidate biomaterials for cancer treatment.

The design of ELRs with a Tt similar to that found under conditions of mild hyperthermia opens the way to this application as, when local hyperthermia is applied, the ELR changes its soluble state to an insoluble one. The ELR therefore aggregates under local mild hyperthermia at the site of action and can be used for cancer therapy. This aggregation increases its accumulation and time of action. Tumour accumulation may be due to the passive trafficking of the ELR due to the EPR effect [27], the increased tumour blood flow and vascular permeability as a consequence of hyperthermia [74], or due to the aggregation itself, which localizes more ELR in the tumour region [75, 76]. Studies with a <sup>14</sup>C-labeled ELR (<sup>14</sup>C ELP1) allowed the degradation rate and accumulation in the tumour to be

studied [75]. The *in vitro* degradation rate upon exposure to murine serum was 2.49 wt.%/day, with similar levels being obtained after intravenous injection into nude mice (2.46 wt.%/day), with a terminal half-life of nearly 9 hours. These data showed a 1.8-fold increase in accumulation for heated tumours when compared with controls. Another study investigated the enhanced accumulation of ELRs at the tumour site due to their thermosensitive behaviour [76]. Consecutive heating cycles were applied to the tumour, resulting in the formation of adherent ELR microparticles in the tumour vasculature in each thermal cycle. In contrast, when the heat was turned off the particles were dissolved, thereby creating a transvascular concentration gradient which pushed the ELR into the tumour space. Aggregate accumulation was monitored by labelling the ELR with fluorescent reporter molecules. This study resulted in a new methodology known as “tumour thermal cycling”, which could potentially be used for targeted delivery to solid tumours.

At a cellular level, the ELR transition can be used to increase cellular uptake of the ELR-drug complex, thus triggering higher delivery levels of the drug. In order to achieve better results, short functional motifs known as cell-penetrating peptides (CPPs), such as penetratin, Bac, Tat and SynB1, were used. These CPPs have been shown to efficiently internalize various molecular loads in order to target the inside of eukaryotic cells [77-79]. Thus, Raucher’s lab developed a thermally responsive ELR with the CPPs penetratin (pen), Tat or Bac fused to the inhibitor c-Myc (H1), which is known to inhibit proliferation in MCF-7 breast cancer cells [80]. The intracellular distribution of the resulting ELR-H1 fused with the different CPPs was evaluated. Bac-ELP-H1 was found to be localized in the nucleus and was the most potent inhibitor of MCF-7 cell proliferation at day 7. In contrast, the Pen- and Tat-modified ELRs were localized in the cytoplasm. As a result, Pen-ELP-H1, which decreases the proliferation rate in MCF-7, had no effect on proliferation up to 11 days of treatment. This study combined the advantages of thermal targeting and delivery of a therapeutic peptide such as c-Myc, thus making this system an improvement on previous studies. Further *in vivo* research using an orthotopic model of breast cancer in C57BL/6 mice to test the ability of the Bac-ELR-H1 construct to target and reduce tumour growth was developed [81]. Treatment involving daily injections of Bac-ELR-H1 during 7 days over a total of 2 weeks, in combination with hyperthermia, reduced the progression of breast tumours by nearly 70%. In treated mice the ELR was able to target the site of the solid tumour, escape from the vasculature, enter the tumour cells and inhibit their proliferation. Similar results were obtained with the same Bac-ELR-H1 construct but this time with glioblastoma in a rat glioma model [82]. An 80% reduction in tumour volume was observed, with complete regression in 80% of animals, when combined with hyperthermia. As a result, tumour-related neurological deficits were delayed and the median survival time doubled.

Additionally, a Bac peptide fused to an ELR was also used by the same group in further studies. Thus, addition of a Bac peptide at the N-terminus and an amino acid fragment from a peptide derived from p21 at the C-terminus led to the construct Bac-ELR-p21 [83]. Under mild hyperthermia conditions, the selected ELR showed a decrease in the

proliferation of SKOV-3 cells in comparison with the thermally unresponsive control. This increase in the anti-proliferation effect was due to the action of p21, which was located in the nucleus for those cells incubated under hyperthermia. Another polypeptide was designed using the same ELR structure but with the CPP SynB1 at the N-terminus and the therapeutic peptide GRG at the C-terminus (SynB1-ELR-GRG) [84]. GRG was designed to interfere with UsnRNP biogenesis by competing for methylation with Sm proteins. *In vitro* assays in HeLa cells showed the localization of the polymer in the cytoplasm. Cellular uptake, proliferation and apoptosis effects derived from the therapeutic GRG peptide were therefore increased by hyperthermia treatment.

Similarly, a targeted CPP-ELR has been used in combination with hyperthermia to deliver small molecule drugs such as doxorubicin [85] or paclitaxel [86]. Thus, doxorubicin was conjugated to the Tat-ELR via a cathepsin-cleavable GFLG spacer to form the construct Tat-ELR-GFLG-Dox as explained above. In a subsequent study [85], the cytotoxic effects of the drug increased 20-fold when the ELR aggregated as a result of the application of mild hyperthermia. Paclitaxel, in turn, was linked to a SynB1-ELR via an acid-cleavable hydrazone linker [86], being SynB1 a CPP that is known to internalize via endocytosis. The hydrazone linker is cleaved at the acidic pH found in lysosomes and endosomes, thereby triggering the intracellular release of paclitaxel. In this study the delivery of paclitaxel was achieved under hyperthermia, and inhibition of proliferation and apoptosis induction was confirmed using MCF-7 breast cancer cells and found to be comparable with the effects of the free drug.

### 3.1.2. Self-assembled ELR particles

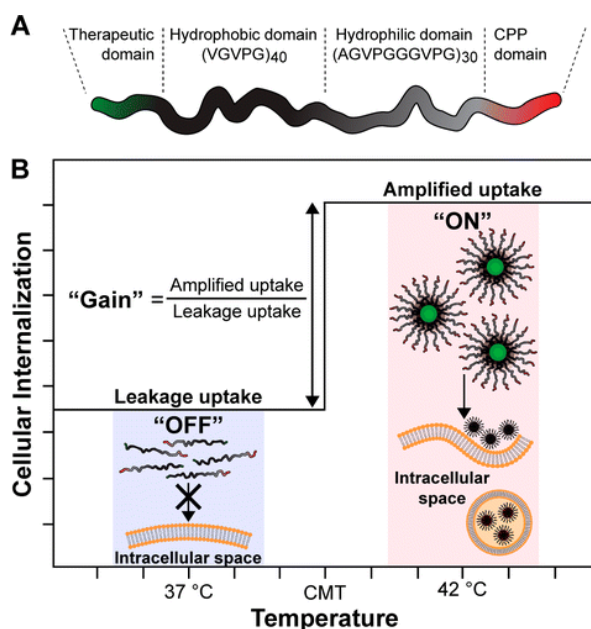
In addition to thermosensitive ELR constructs that aggregate, even reaching depot status, self-assembled ELR-based architectures are also attractive for the biomedical field. In this sense ELRs are usually designed as block copolymers comprising a hydrophilic and a hydrophobic part being able to self-assemble, thus leading to the formation of well-defined nano-sized particles. When the polymer is solubilized in aqueous solution and is heated above its Tt, the ELR chains orientate in a way that triggers the formation of core-corona structures, with the corona comprising hydrophilic blocks and core hydrophobic blocks. The micelle or hollow vesicle shape resulting after self-assembly can be changed by varying the ELR block arrangement and length [59]. Studies with different ELR constructs containing glutamic acid and alanine blocks confirmed this possibility, thereby highlighting the high versatility of ELR-based diblocks. ELR diblocks in which the hydrophobic part possesses a Tt > 42 °C under local mild-hyperthermia conditions have been widely studied due to their potential use in cancer therapy [87].

Self-assembled ELR nanoparticles can acquire different functionalities upon the incorporation of recognition motifs such as RGD, NGR or Fn3 peptides in the hydrophilic block. The RGD peptide acts by increasing the avidity of the copolymer for its ligand ( $\alpha$ IIB integrin in activated platelets and the  $\alpha$ v $\beta$ 3 integrin in healthy angiogenic tissue in addition to tumour vasculature) while maintaining its Tt and ability to

self-assemble, thereby constituting a thermally tunable switch for the ELR in K562 leukaemia cells [88]. NGR is known to bind to CD13 isoforms expressed in tumour vessels and has been widely used for tumour targeting. This peptide was bound to ELR diblocks in a series of constructs with different hydrophilic-to-hydrophobic ratios [89]. At ratios of between 1:2 and 2:1, monodisperse spherical micelles were found with the peptide pointing outwards. In addition, the critical micelle temperature (CMT) could be controlled by varying the length of the lower Tt block. This study was the first demonstration of temperature-triggered multivalency in which ELR diblocks with a functional peptide maintain their ability to self-assemble and target tumour cells, with self-assembly into micelles enhancing the local density of the functional peptide on the corona.

The fibronectin type III domain (Fn3), which binds the integrin  $\alpha v \beta 3$ , has also been fused to ELR diblocks [90]. The self-assembly behaviour of the resulting constructs was studied through the bioproduction of ELRs with different hydrophilic and hydrophobic block lengths. The result was an increase in the uptake of ELR with Fn3 in comparison with the unimers in a transfected human leukaemia cell line overexpressing the integrin  $\alpha v \beta 3$  (K562/ $\alpha v \beta 3$ ). In a later study, a qualitative breakthrough in the design of ELR diblocks fused to functional peptides was accomplished. Thus, Chilkoti's group constructed an ELR-based drug carrier termed nanopeptifier, that was able to improve cellular uptake by controlled exposure of CPPs, which toggled in response to the application of external mild hyperthermia (**Figure 3**) [91]. The construct (Arg8-ELRBC-cBH3) was genetically engineered to possess a CPP (Arg8) at the N-terminus and the therapeutic payload peptide cBH3 (derived from the proapoptotic Bak protein) located at the C-terminus. The therapeutic cBH3 peptide was separated by an RVR peptide linker cleavable by furin and cathepsin B proteases. The nanopeptifiers are soluble at physiological temperature (37 °C), whereas under of mild hyperthermia conditions (42 °C) they self-assemble into spherical micelles displaying a high density of CPP on their corona and with cBH3 located in the micelle core. Although some uptake of the nanopeptifier occurs at 37 °C, when the temperature is increased to 42 °C this cell uptake increases dramatically. Once the complex is internalized, the action of either furin (localized in the trans-Golgi) or cathepsin B (early and late endosomes and lysosomes) favours release of the cBH3 load. Increases of up to 130 arbitrary cell fluorescence units were found to induce the therapeutic effect of cBH3. Its therapeutic action was assessed by activation of caspase-3, which is involved in apoptotic processes. Thus, the nanopeptifier was able to create a cytotoxic switch that induces apoptosis only when it is in the micelle conformation.

In addition to ELRs genetically designed as block copolymers, a series of smart ELR-PEG block constructs have been synthesized chemically [92]. The specific strain promoted alkyne-azide cycloaddition (SPAAC) reaction was used to build the copolymer. Constructs with different ELR and PEG lengths were accomplished by varying their architecture (linear or miktoarm). The result was conservation of the thermosensitive nature of the ELR, which promoted self-assembly into micelles. The ability of these constructs to encapsulate a hydrophobic fluorescence



**Figure 3.** Design and function of an ELR diblock nanopeptifier. (A) Each nanopeptifier is formed by a therapeutic payload, an ELR diblock and a CPP. (B) At physiological temperature (37 °C) “off state”, nanopeptifiers are soluble displaying a single CPP on their hydrophilic terminus. At mild hyperthermia conditions (42 °C) “on state” the nanopeptifiers self-assemble into spherical micelles, displaying a high density of CPPs on the micelle corona. The increases CPP density amplifies the cellular uptake [91].

dye was shown, thereby demonstrating the potential of these ELR-PEG hybrid copolymers for the encapsulation of hydrophobic drugs.

The above examples reflect the potential of ELRs as thermoresponsive materials, which are of growing interest in the biomedical field. Their recombinant nature, which allows a total control of their architecture, and their self-assembly properties open the way to new features such as thermal switching, which can even be used to direct cellular uptake. All these studies involve new applications in diseases such as cancer and are therefore a promising field for ELR-based biomaterials.

### 3.2. Drug-delivery systems based on pH-sensitive ELRs

pH-Sensitive drug-delivery systems have received increasing interest in recent years as pH plays an important role in many biological process and some diseases such as asthma, peptic ulcer, heart disorders, hypertension and cancer [93]. For example, the pH found in healthy tissue or in the blood is different to that found in tumours due to tumour hypoxia and the production of substances such as lactic acid resulting from anaerobic metabolism and ion channel changes [94, 95].

Given these differences in pH between normal tissue and the tumour microenvironment, Callahan *et al.* proposed the first pH-sensitive ELR-based polypeptide micelle that disassembles at the lower pH found in solid tumours. Based on previous work in which these authors demonstrated that ELR block copolymers with different lower critical solution temperatures (LCSTs) are able to form micelles over a



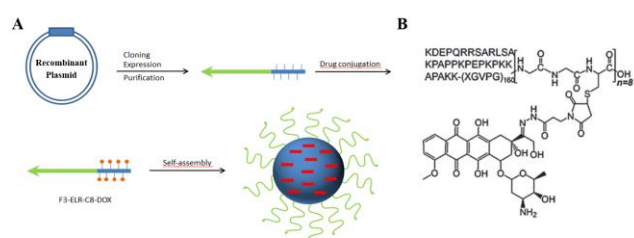
specific temperature range [89], ELR<sub>BCS</sub> containing histidine-rich hydrophobic blocks were designed with the aim of creating pH-sensitive nanoparticles that self-assemble at pH 7.4 and disassemble at pH 6.4 [24, 96]. Thus, they synthesized two different ELR<sub>BCS</sub>, containing a combination of hydrophobic (pH-insensitive block, to form the corona of the micelles) and hydrophilic blocks (pH-sensitive blocks, to form the core of the micelles). [VG7A8]-80/[VH4]-100 was selected as it was found to be the only ELR<sub>BC</sub> that formed stable nanoparticles at 37 °C. A pH-insensitive ELR [VG7A8]-64/[V]-120 was selected as negative control in order to verify the ability of the pH-sensitive micelle formed by [VG7A8]-80/[VH4]-100 to respond to changes in pH.

The ELR<sub>BCS</sub> were examined in nude mice bearing HCT-15 human colon adenocarcinoma xerographs. Both polymers maintain the beneficial behaviour of high MW drug-delivery nanoparticles, with [VG7A8]-80/[VH4]-100 presenting a lower accumulation in healthy tissues and greater affinity for the lower pH of the tumour environment. It was found that [VG7A8]-80/[VH4]-100 shows a more homogeneous spatial distribution and greater penetration within the tumour tissue compared to pH-insensitive ELR<sub>BC</sub> micelles based on [VG7A8]-64/[V]-120. The histidine-rich ELR<sub>BC</sub> micelles are able to undergo a pH-triggered disassembly that means the individual polymer chains increase their diffusion capacity and can penetrate inside the tumour. However, pH-insensitive micelles tend to accumulate around the tumour space as they cannot respond to lower pH, and cannot therefore undergo pH-dependent disassembly, thus meaning that the rate of accumulation in the extravascular space increases. This new pH-responsive mechanism suggests the potential utility of this system for improving drug delivery to, or the imaging of, solid tumours.

Another strategy is based on the design of a different structure in which pH-dependent delivery is related to pH-labile amino acid sequences. In order to improve the results obtained by MacKay *et al.* in a previously reported study [54], a new strategy was proposed by Hu *et al.* Thus, these authors reported the first ELR genetically fused to a tumour homing peptide, F3, fused to the same structure proposed previously by MacKay *et al.* and also carrying a cysteine-rich fragment (Gly-Gly-Cys)<sub>8</sub> (C8) with the aim of creating a bioactive and well-defined polypeptide F3-ELP-C8, which was conjugated with doxorubicin (DOX). F3 is a 31-amino acid sequence that specifically attaches to nucleolin, which is present on the surface of activated lymphocytes, angiogenic endothelial cells and many tumour cells [97, 98]. The C8 segment was selected to bind multiple DOX molecules to the Cys residues of the F3-ELR-C8 via an acid-labile hydrazine linker (3-maleimide propionic acid hydrazine (BMPH)) to form F3-ELR-C8-DOX (**Figure 4**). They demonstrated that DOX has a faster delivery at lower pH (5.0) than at pH 7.4 (physiological pH), where most of the DOX molecules are attached to the conjugate. This result reveals that the hydrazone bond is stable at the pH of blood and healthy tissues but breaks at lower pH. The pH-dependent release of DOX from the conjugates suggests endolysosomal drug delivery once the nanoparticles have been taken up by the cells, which is related to the pH-responsive behaviour.

*In vitro* studies demonstrated that F3-ELR-C8-DOX nanoparticles bound to the cell surface earlier and exhibited

more drug accumulation in the cell cytoplasm and nucleus than ELR-C8-DOX, thereby demonstrating the important role that F3 plays in the cell interaction. This result was confirmed by way of *in vivo* experiments in mice with a C26 tumour model, in which F3-ELR-C8-DOX was found to have a longer half-life than free DOX and ELR-C8-DOX, a lower accumulation in cardiomyocytes and did not cause histological damage in heart tissue. This is an important point due to the fact that cardiocytotoxicity is often the worst side effect of DOX use. With regard to its efficiency in reducing the tumour volume, the results obtained thirteen days after treatment demonstrated that it exhibits better antitumour efficacy and increased animal survival compared with the control ELR-C8-DOX. Furthermore, histological analyses showed a higher apoptotic effect, which is related to the pH-sensitivity and local tumour distribution of the nanoparticles around the tumour cells [25].



**Figure 4.** Schematic representation of the synthesis of F3-ELR-C8-DOX nanoparticles, showing (A) the synthesis-conjugation of F3-ELR-C8-DOX and (B) the molecular structure of this drug delivery system. Adapted with permission from Hu *et al.* [25].

#### 4. THERAPEUTIC ELR DEPOTS AND HYDROGELS

The smart behaviour and temperature sensitivity of ELRs can be exploited in such a way as to control their aggregation both *in vitro* and *in vivo*, thus paving the way to advanced medical applications such as drug depot formation for tissue regeneration, neuroinflammation, cancer and diabetes.

As a general rule of thumb, these ELRs have been designed to possess a T<sub>t</sub> below the physiological temperature of 37°C. The ELRs are solubilized and injected at the desired site of action as soluble ELRs (unimers), where they immediately coacervate and form the depot as a result of the temperature. Thus, the direct application of ELRs at the target site helps to extend local drug exposure time while minimizing systemic side effects [31].

As stated above, one of the main applications for ELR depots is in regenerative medicine. Thus, research in knee joint regeneration has investigated the use of a thermally responsive ELR that could spontaneously aggregate upon injection to form a “drug depot”. The *in vivo* biodistribution in a rat model showed that the aggregated ELR had an approximately 25-fold increase in intra-articular half-life in comparison with the non-aggregated ELR. This finding made it appropriate for sustained delivery of protein drugs for treating osteoarthritis [99]. More recent work in the treatment of post-traumatic arthritis involved the development of a cross-linked ELR and the evaluation of intra-articular delivery of IL1Ra (IL1 receptor antagonist) and TNFR1I (TNF $\alpha$  inhibitor). The results showed a sustained delivery of both inhibitors and a corresponding

decrease in osteoarthritic damage caused by the disease [100]. As regards the development of carriers for the delivery of osteogenic drugs, such as bone morphogenetic protein (BMP)-2 and BMP-14, a VPAVG-based ELR that forms stable and monodisperse depots was used to encapsulate both therapeutic proteins separately. The *in vitro* release profile was found to be fast during the first 24 hours followed by a slower release over 14 days. Trans-differentiation of C2C12 mouse myoblast cells towards osteoblasts in *in vitro* cell culture experiments demonstrated the efficient delivery of both BMPs. These results suggest the application of this system in the delivery of many other drugs [101].

ELR depots can also be applied in other pathological targets such as neuroinflammation in order to improve the inflammatory symptoms. In studies to treat local neuroinflammation at the dorsal root ganglion using ELR depots, a sevenfold increase was found in perineural half-life in comparison with the soluble ELP. This result provides evidence of high and sustained levels of therapeutics at the dorsal root ganglion while minimizing systemic exposure [102]. Another interesting approach in the growing field of neuroinflammation is the use of depots comprising ELRs chemically conjugated to curcumin (a TNF $\alpha$  antagonist) via a degradable carbamate linkage [103]. The resulting ELR maintained the thermally-triggered depot formation after curcumin conjugation and hence the bioactivity against TNF $\alpha$ . When injected proximal to the sciatic nerve in mice via intramuscular (i.m.) injection, ELR-curcumin underwent a thermally triggered soluble-insoluble phase transition, thus leading to *in situ* formation of a depot and resulting in a fivefold higher level of the drug at four days post-injection when compared with the free drug and an up to sevenfold decrease in plasma levels.

ELR-radionuclide conjugates have been explored as therapy for the local treatment of tumours. In this case, an ELR was designed and conjugated to the therapeutic agent iodine-131 and delivered intratumourally. An increase in the residence time in the tumour, together with a longer protection of the radionuclide from halogenation, when compared with a soluble ELR, was observed [104]. Although these ELR-iodine-131 depots improved survival and tumour regression in murine xenograft tumours, they were unable to achieve complete tumour regression. Further optimization was performed to improve this aspect [105]. This optimization was achieved after investigating the effect of a wide range of parameters that influence tumour retention and modulate the physical properties of the depot, such as molecular weight, amino-acid composition and the tyrosine-rich peptide appended to the carboxy-terminus for radiolabelling. The radionuclide-polypeptide resulting from this study was able to self-assemble to form seeds that led to prolonged intratumoural retention, tumour growth delay in 100% of tumours and cure in more than 67% of tumour-bearing animals in two human xenografts of hypopharyngeal and prostate cancer cells (FaDu and PC-3 respectively).

ELR depots can also be used to deliver the glucagon-like peptide-1 (GLP-1), with which they form a 50 kDa polymer that has shown great promise for the treatment of type 2 diabetes with a glucose-dependent insulinotropic effect. Following this strategy, ELR depots were genetically

designed with the incorporation of GLP-1 flanked by protease cleavage sites [106]. The delivery of GLP1 from the depot and activation of the GLP-1 receptor were subsequently confirmed. The resulting depot was termed a "protease operated depot" (POD). The novel contribution of this system lies in the reduction in the number of injections since a single injection of GLP1-ELR depot reduced blood glucose levels in mice for up to 5 days, which is 120 times longer than for an injection of the native peptide.

Different ELRs hydrogels have been designed for biomedical applications with an amphiphilic block co-recombinamer structure that spontaneously self-assemble to form gels which physical crosslinking relying on hydrophobic interactions between apolar blocks. These systems mimic *in vivo* cellular environment enabling *in vitro* studies of cell-ECM interactions [107]. Being made up of ELRs constitutes an advantage because of their tunable mechanical properties, well defined biological and chemical activity and, if necessary, a controlled topography [108, 109]. These features made them potentially useful as scaffolds for tissue engineering. The only drawback with such non-covalent bonding is that it sometimes results in inadequate mechanical properties or low stability of these structures. To overcome these disadvantages and to stabilize ELR-based hydrogels, a bioinspired peptide such as the silk fibroin domain [110], which is able to establish more robust physical cross-links, has been incorporated into the ELR backbone to give rise to the family of silk-elastin-like polymers (SELPs). This family of genetically engineered protein block copolymers is made up of silk fibroin domains, which follow patterns such as GAGAGS, and elastin-like blocks of the type GXGVP, where the guest amino acid "X" can be any amino acid except proline. The silk-like block tends to self-assemble into an anti-parallel  $\beta$ -sheets secondary structure, thereby providing physical crosslinking sites for the SELP polymeric systems that enable access to physical hydrogels with higher thermal and chemical stability and mechanical tunability [111]. Exhaustive studies have been conducted into the modulation of SELP properties, especially by varying the silk-elastin ratio [112], molecular weight [113] and X residue in the elastin sequence [114]. SELPs can act as an injectable gel-forming material for controlled drug and gene release of DNA under physiological conditions. Initial research by Gustafson *et al.* [112] studied the release from hydrogels comprising various SELPs with different silk-elastin ratios and loaded with adenoviruses carrying reporter genes for head and neck tumours. The biopolymer SELP-815K, with eight silk and fifteen elastin units and a lysine (K)-modified elastin, had the highest ratio of silk content and showed the highest gene expression compared with the others studied (SELP-415K and SELP-47K). The tumour to liver expression ratio was up to 55-fold higher for the SELP-815K system compared to viral injection without the polymer, thus proving the utility of these matrices for gene therapy in head and neck cancer. Subsequent studies [115] with SELR matrices consisting of the same structured SELP (SELP-815K) loaded with an adenovirus containing both thymidine kinase-1 and luciferase genes was performed to check the efficacy and safety of tumour therapy. The SELP-815K delivery system was injected intratumourally into a mouse model of head and neck cancer and the gene transfection efficiency, duration of

transgene expression and biodistribution analyzed using the bioluminescence provided by the luciferase gene. SELP-controlled therapeutic agent delivery at the tumour site resulted in greater confinement, with no detectable spread to the liver, which contrasts with the results obtained for intratumoural injection of adenoviruses in saline, in which 50% of animals showed clear liver dissemination. An approximately fivefold greater reduction in tumour size was observed in the adenovirus-SELP 815K system compared with the results obtained for saline/adenovirus treatment, thus demonstrating the anticancer efficacy of these hydrogel systems [115].

SELP structures modified to introduce sensitivity to matrix-metalloproteinases (MMPs) were designed to increase the local delivery of bioactive agents to solid tumours in which MMPs are overexpressed. A hydrogel that is degradable by MMPs was produced and its protease sensitivity tested *in vitro* by incubation with different metalloproteinases (MMP-2 and MMP-9) to assess complete removal from the potential administration site and the correct release of bioactive agents. This was checked by measuring the mechanical properties of the hydrogel and the fluorescence of polystyrene beads released, with the results suggesting the ability of these systems to respond to local changes in MMP concentration when injected intratumourally [116]. *In vivo* degradation assays were subsequently performed in the presence of solid head and neck tumours. A significantly higher degradation was observed for MMP-responsive SELPs than for SELP-815K, whereas degradation of their hydrogel devices is polymer structure dependent, thus demonstrating the tunable nature of MMP-responsive SELPs for matrix-mediated gene delivery [117]. In light of the above, we can conclude that this type of biomaterial is especially useful for gene therapy as polymeric hydrogels control DNA release, allow localized delivery to specific or damaged tissue and protect DNA from degradation, thereby improving the safety and efficacy of gene delivery.

ELRs have been used to form ELR-collagen hydrogels in order to optimize and reinforce the features of pure collagen hydrogels. One of the most widely applied hydrogels for local drug delivery applications is that comprising collagen, despite the fact that it lacks the rigidity required for tissue engineering and, more importantly, that it is rapidly degraded by collagenases to form products that elicit inflammation. Anderson and co-workers have prepared ELP-collagen hydrogels physically cross-linked by hydrogen bonding and hydrophobic associations that displayed superior physical and mechanical properties than pure collagen scaffolds, which was attributed to a homogeneous network structure with additional reinforcement due to the ELR aggregates [118]. Subsequent studies concerned the release behaviour of the ELR-collagen hydrogels prepared in the presence of doxycycline hyclate and loaded with different amounts (1-5%, w/w) of this commonly used broad-spectrum antibiotic in periodontal or acne therapy. The ELR-collagen composite showed slower doxycycline release compared to the collagen hydrogels, probably due to a smaller swelling ratio, with a gradual time-dependent drug release over a period of 5 days irrespective of the hydrogel composition. The collagen and ELR that make up the hydrogels did not interfere with the bioactivity of the drug and the released doxycycline was effective against four

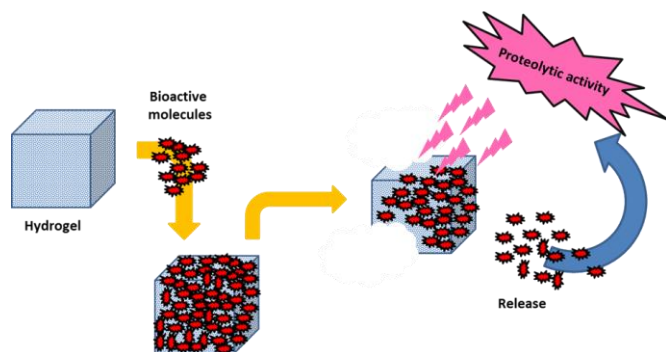
bacterial strains (*E. coli*, *P. aeruginosa*, *S. sanguinis* and methicillin-resistant *Staphylococcus aureus*), thus meaning that this biocompatible composite hydrogel allows sustained release of an antibiotic at the local site with the potential to minimize post-surgical infections and support wound healing.

A novel ultrasound-based method has been used to generate physical ELR hydrogel scaffolds for the local delivery of proteins and antibiotics. These hydrogels are formed by sonicating an aqueous solution of the recombinamer [(VPGVG)<sub>120</sub>] for a short period of time at low temperature (4°C) and subsequently incubating at physiological temperature for an extended period of time, in the presence of bovine serum albumin and doxycycline hyclate, which were added to the sonicated ELR to be enclosed. The mechanism suggested for physical cross-linking involves a hydrophobic interaction between polymeric chains after ultrasonication. The release of BSA and doxycycline from the scaffold was studied at different temperatures, above (37 °C) and below (25 °C) the transition temperature (T<sub>t</sub>) and with different loading concentrations of both bioactive molecules. Both BSA and doxycycline showed a gradual time-dependent release that is affected by factors such as drug molecular weight and loading concentration. The highlight of this approach is the ease of formation of ELR hydrogels without needing to add chemical crosslinking agents and a release governed by different factors such as temperature or drug loading dose, thereby suggesting potential applications in controlled drug delivery [119].

To increase the structural stability and mechanical properties of ELR hydrogels, which may be necessary for some applications, chemical hydrogels were synthesised by chemical, enzymatic or photo-irradiated cross-linking [120-123]. In addition, a chemical hydrogel to be used as an injectable “click” system has been developed starting from a pair of elastin-like recombinamers suitably chemically modified in such a way that a covalently crosslinked hydrogel is formed under physiological conditions, in a short period of time, and once the two components are in contact. The inclusion of an amino acid that can subsequently be modified to contain adequate functional groups for covalent cross-linking is necessary for this purpose. The introduction of a lysine residue as a guest amino acid distributed along the peptide chain allows ELRs to be modified at the ε-amino group to bear the reactive groups necessary for the “click reaction” [11]. To this end, ELRs were modified to carry azide and cyclooctyne groups capable of generating interchain bonds through triazol heterocycles via a copper-free 1,3-dipolar cycloaddition. The resulting hydrogel system is biocompatible and permits the homogeneous entrapment of drugs and bioactive molecules [124]. Nanogels were prepared at different temperatures by mixing dilute solutions of the aforementioned click-reactive ELRs with the aim of understanding the relation between the macroscopic properties of the resulting ELR-click gels and the initial stages of the chemical crosslinking process, which involve fractal modes of gel formation. Different topographies were obtained for the nanogels prepared at temperatures below and above the molecular transition temperature of the ELRs, thus implying that the cross-linking temperature determines the microstructure of the final hydrogels [124]. In a subsequent study, the previously developed hydrogels

obtained by the catalyst-free click reaction of elastin-like recombinamers were used to fabricate a new class of vascular stents coated with this biocompatible material. Thus, the bare metal stent is embedded into an injection mold that is filled with the ELR gel. In order to avoid atherosclerotic plaque formation that induces re-stenosis, stent endothelialization was performed in a bioreactor under dynamic pressure and flow conditions. Once endothelialized, the ELR-covered stents constituted an endovascular device with a physical barrier against atherosclerotic plaque, biocompatibility, no inflammatory response and physiological haemocompatibility, thus paving the way to a future generation of eluting stents [125].

One of the most advanced hydrogel-based constructs for drug delivery is made up of enzymatically cross-linked ELRs that are able to release bioactive substances under specific stimulus from the environment. As has been described previously [126], there is a correlation between the elastolytic activity and pathological conditions such as atherosclerosis, cystic fibrosis or chronic wounds, amongst others. In this regard, Bandiera *et al.* have designed an *ad hoc* ELR-based hydrogel system that carries both chemical cross-linking and protease-sensitive domains (Figure 5). ELR crosslinking is performed via transglutaminases in the presence of a recombinant green fluorescent protein (eGFP) that is embedded during the gelation process. The hydrogels formed were exposed to the proteolytic activity derived from *P. aeruginosa* and human polymorphonuclear leukocytes (PMNs), which triggered the gradual release of eGFP from the matrix as it was degraded. In short, a drug delivery platform capable of interacting with biological systems that can sense a chemical abnormality in damaged tissues and respond by releasing the therapeutic agent to interfere with the pathological process, thus leading to the inhibition of potentially harmful processes, has been designed [127].



**Figure 5.** Bioactive molecule delivery triggered by elastolytic activity of pathological processes and drug release leading to proteolytic activity inhibition. Adapted with permission from Bandiera *et al.* [127].

An alternative approach to chemical hydrogels is that designed by Chilkoti *et al.*, which consists of a reversible hydrogel that is formed under mild oxidative conditions by intermolecular disulfide chemical crosslinking of ELRs containing periodic cysteine residues along their chain. The rapid gelation of these custom-designed ELRs under physiological conditions upon addition of a low concentration of hydrogen peroxide (0.3 wt %) makes them useful as an injectable gel-forming material. Preliminary *in vitro* studies of the release profiles for BSA protein indicated

a sustained release pattern, thereby demonstrating the advantage of hydrogel formation for stable entrapment of a BSA payload with respect to the control ELR-based coacervates. Indeed, after 4 hours of incubation only 20-40% of release was detected for ELR hydrogels crosslinked via cysteine residues, whereas complete release was observed for ELR coacervates over the same time period. The *in vivo* intratumoural injection of *in situ* gelling radiolabelled ELR to mice bearing human pharynx squamous xenografts showed higher and more prolonged tumour retention (greater than 40% of the payload) and homogeneous distribution across the entire tumour, in contrast to ELR coacervates, for which retention was less than 10%. This enhancement of radionuclide retention makes these systems suitable for local cancer therapy of tumours refractory to surgical removal.

## 5. SMART DRUG-DELIVERY SYSTEMS NOT INVOLVING ELRS

Numerous drug-delivery systems other than those based on elastin have been designed to achieve therapeutic efficiency at the application site and to overcome the aforementioned limitations in classical therapeutic disease treatments [2]. The most common such drug-delivery systems comprise new macromolecular carriers with a defined structure and properties that can respond to environmental stimuli with a pronounced change in their structure. To the best of our knowledge, these drug-delivery systems can be made from a wide variety of structures involving both polymeric and non-polymeric building blocks [32]. One interesting improvement is the use of environmentally sensitive polymers that are able to respond to external stimuli such as temperature, pH, ion concentration or electric fields, among others. A great variety of drug-delivery vectors have been designed using the above-mentioned building blocks, including micelles, liposomes, polymeric and protein drug conjugates or inorganic nanoparticles, each with different advantages [3, 4, 128].

Amongst other polymeric drug-delivery systems consisting of natural proteins, those based on silk and collagen, which have been mentioned above in combination with ELPs, can be highlighted.

Silk proteins has been used with great success in devices for cell culture and tissue regeneration and have been approved by the FDA due to their versatility and biocompatibility [129, 130]. Numata *et al.* have proposed a genetically engineered silk-based ionic pDNA complex with a poly(L-lysine) that interacts with pDNA and has a higher content of tumour-homing peptide (THP) than in their previous studies in this field with the aim of improving its transfection efficiency and specificity for tumour cells [131]. Two different THPs were used with three different cell lines, namely MDA-MB-435 melanoma, MDA-MB-231 metastatic human breast tumour cells and MCF10A non-tumour mammary epithelial cells as control. The results showed that the pDNA complexes containing THP showed greater specificity against tumour cells compared with lipofectamine and the pDNA complex without THP used as control. In addition, a comparison of these THPs showed that the pDNA complex with F3 as THP appears to be the best candidate for target delivery due to its lower cytotoxicity as a result of the

ability of pDNA to complex with Lyp1 to transfect non-tumorigenic cells [132].

A very recent study describes the fabrication of silk fibroin (SF) microparticles for the delivery of small-molecule drugs for the treatment of arthritis by increasing the local residence time after intra-articular injection of the drug-loaded particles. To this end, microparticles were formed from a solution of a mixture of SF and the fluorophore Cy7, which establish mutual hydrophobic interactions, using previously described methods but with different ratios of the two components. Microparticle formation was confirmed by scanning electron microscopy, finding a wide range of particles that were nevertheless suitable for intra-articular injection. *In vitro* release studies revealed a faster release in the case of particles containing lower amounts of SF, thereby suggesting a negative effect of Cy7 on particle stabilization by diminishing intermolecular SF interactions. However, the release of Cy7 exceeded 10% of the loaded molecule over the 160 h that the experiment lasted. Additional experiments were performed in rats to evaluate intra-articular retention of the fluorophore using an *in vivo* imaging instrument. However, as Cy7 scattering and decay resulted in difficulties in measuring the evolution of the model compound over space and time, the decay half-life was used as the study parameter and showed an increase from 12.6 h ( $\pm 2.2$  h), in the case of the SF-Cy7 solution without particle-forming treatment to 43.3 h ( $\pm 14.4$  h) for the SF-Cy7 microparticles. Moreover, localization was improved with the novel treatment, thereby reducing the exposure of adjacent tissues to the fluorophore. All these findings suggest the need for further studies regarding this approach to intra-articular drug-delivery systems [133]. However, some problems arise from this work as the particles were not loaded with an actual drug or with non-hydrophobic molecules. Furthermore, fluorophore decay half-life might not be an accurate measurement of drug retention, although a slower decay was demonstrated when compared to SF-Cy7 solutions.

Collagen can be shaped into a great variety of forms for use as scaffolds or drug-delivery systems in numerous medical applications [134]. In order to prepare novel collagen (CG) scaffolds for use as drug-delivery systems that are likely to respond to an external stimulus, they were loaded with paramagnetic iron oxide nanoparticles (NPs) and subsequently covalently cross-linked by way of a dehydrothermal cross-linking treatment to obtain porous matrices. Different characterization techniques were used to gain information about the scaffolds themselves (e.g. SEM) and the amount of NPs included in them (thermogravimetric analysis). Fluorescein was subsequently used as a model compound to study the release by fluorescence spectrometry. The results showed a very low diffusion of fluorescein after immersion of the CGNP scaffold in water during 60 minutes when compared to the CG control. Furthermore, a controlled release of the compound was observed upon application of an external magnetic field to the NP-loaded system, and said release could be switched on and off by switching the magnetic field on and off. Finally, a cell viability assay was

performed with the scaffold alone. This study showed a reduction of about 10% in cell survival in the case of both CG and CGNPs when compared to the negative control, which could be considered as a promising result for further application of this system [134].

As regards polymeric carriers for encapsulated drugs, numerous types of synthetic materials are currently employed, including poly(lactic acid) (PLA), poly( $\epsilon$ -caprolactone) (PCL), and poly(lactide-*co*-glycolide) (PLGA) [4]. Indeed, poly(lactic-*co*-glycolic acid) (PLGA) has been approved by the FDA for use in several therapeutic devices because of its ability to biodegrade, its biocompatibility and its multi-responsive behaviour [35, 135]. PLGA has found numerous applications, for example, in tissue engineering, medical and surgical devices and drug delivery [35]. Herein we focus our attention on applications of importance in drug delivery. Thus, numerous chemotherapeutic agents, such as docetaxel, paclitaxel, doxorubicin and other peptides, can be delivered using PLGA-based delivery systems [35]. Moreover, PLGA immunoconjugates can be applied as targeted anti-cancer systems. Thus, Parveen *et al.* designed a PLGA-chitosan-PEG (polyethylene glycol) conjugated with paclitaxel, and *in vitro* studies carried out in retinoblastoma, invasive breast cancer and pancreatic cell lines demonstrated that PLGA-CS-PEG nanoparticles have a more potent antiproliferative effect and ability to induce cell cycle arrest than PLGA nanoparticles and free paclitaxel. These results suggest that the new conjugate with PEG and chitosan could be a future candidate for targeted delivery of chemotherapeutic agents [136]. Another targeted delivery system could be applied in the field of diagnostic imaging, as proposed by Niu *et al.* and Schleich *et al.* in recent studies [137, 138].

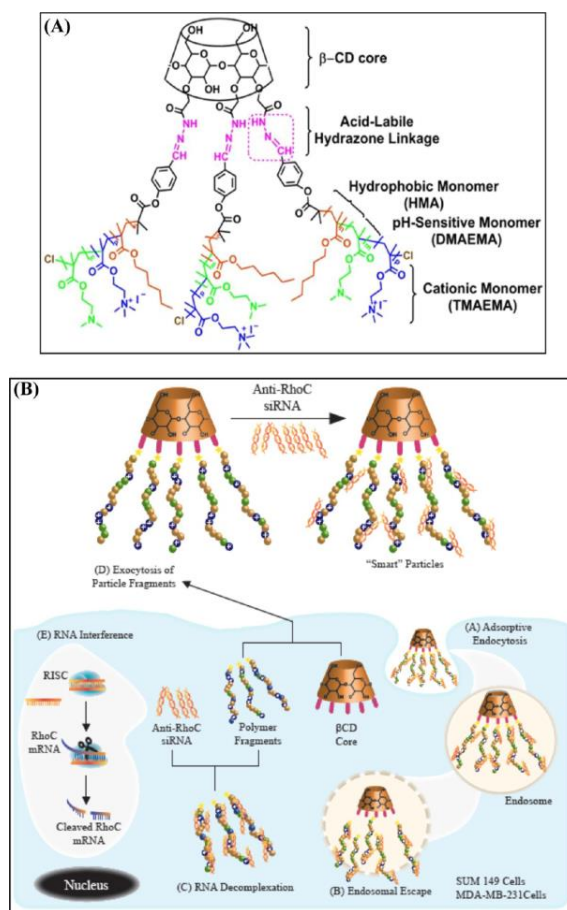
Collagen membranes are excellent candidates for guided bone regeneration (GBR) in combination with bone grafting materials (BGM) as they are subsequently absorbed, thereby avoiding the need for second surgery to remove the membrane [139]. However, as mentioned earlier, this system undergoes fast degradation, thereby limiting the possibility of the controlled release of drugs such as dexamethasone (DEX), which is an excellent osteoinductive growth factor [140-143]. Piao *et al.* have overcome this problem by incorporating PLGA, which is an excellent controlled release carrier that allows an efficient release of the drug [144, 145]. *In vitro* studies in MG63 cells revealed that release of DEX from PLGA on the collagen surface is faster by day 5 but can be controlled for four weeks. These results also show that PLGA improves the attachment of cells in the collagen membrane conjugate compared with the collagen membrane also. *In vivo* analyses have been carried out in rat models with a calvarial bone defect. The results obtained after eight weeks showed that the collagen membrane coated with has a better ability to induce osteogenesis than the membrane without DEX [146] due to controlled release from PLGA into the collagen membrane. DEX induces cell proliferation, differentiation, extracellular matrix secretion and tissue formation [147].

A new approach, namely the use of PLGA as a coating for drug-eluting stents, has recently been gaining importance, especially as atherosclerosis is one of the main problems related to stent implantation. In initial studies, Ganaha *et al.* had shown the ability of a combination of PLGA microspheres with a stainless steel stent and angiostatin to decrease plaque progression and avoid re-stenosis [148]. The favourable characteristics of PLGA, such as biocompatibility, biodegradability and its non-toxic properties, offer a wide range of possibilities for application in the development of new drug-delivery systems and other biomedical applications.

A novel proposal for the treatment of invasive breast cancer, consisting of a pH-sensitive polymer conjugated with siRNA fragments, was developed by Kaushal *et al.* This system comprises a  $\beta$ -cyclodextrin core linked to the designer polymer by an acid-labile hydrazone bond, with the polymer itself comprising a hydrophobic monomer (HMA, hydrophobic hexyl methacrylate) connected to a pH-sensitive monomer (DMAEMA, 2-(dimethylamino)ethyl methacrylate) and, in turn, to a cationic monomer (TMAEMA, *N,N,N*-trimethylaminoethyl methacrylate) (Figure 6). This latter cationic domain is attached to the anti-RhoC siRNA by electrostatic interactions between the amine groups of the polymer and the phosphate groups of the siRNA. *In vivo* studies in SUM149 and MDA-MB-231 cell lines revealed that the resulting nanoparticles can be internalized into the cells, then leak from the endosome to subsequently deliver their siRNA load. Expression of the RhoC gene is selectively diminished by 90-100% and, as a result, the invasion, motility and migration of both SUM149 and MDA-MB-231 are considerably reduced. Other targets could be explored in the future with the aim of increasing the diversity of these smart nanoparticles combined with siRNA to reduce cell invasion and motility and induce an ability to reduce other types of tumour cells [149].

Other non-polymeric drug-delivery carriers are defined by non-repetitive amino acid motifs with an ability to form tertiary or quaternary structures, including vault proteins [33] and viral proteins, amongst other [34].

One example of vault proteins was proposed by Buehler *et al.* with the aim of improving their previous proposals for vault-drug delivery [33]. In this case, they formed a new class of nanoparticles resulting from the covalent attachment of NS5A1-31 amphipathic  $\alpha$ -helix (a peptide derived from hepatitis C virus) to MVP, with these NPs showing a self-assembled protein shell with a lipophilic core that is able to retain small molecules for subsequent controlled drug delivery. Bryostatins were subsequently incorporated into the AH-vault carrier with the aim of developing an anti-HIV/AIDS agent. Previous studies showed that bryostatin 1 incorporated into lipid nanoparticles can activate latent HIV and can be triggered against CD4+ T cells [150]. The resulting AH-vault-bryostatin 1 was found to activate latent HIV provirus in J-Lat 10.6 cells in a dose-dependent manner compared with empty AH-vaults, and was also able to prompt cell-surface expression of CD69 at similar concentrations to those required to activate latent HIV [151, 152]. *In vivo* analyses in C57/bl6 mice revealed that over 90% of CD4+ had been induced to express CD69 24 h post-injection of AH vault with bryostatin 1, thus highlighting



**Figure 6.** (A) Chemical structure of the pH-sensitive,  $\beta$ -CD-g-P(HMA79-co-DMAEMA33-co-TMAEMA48)4.8 polymer. (B) Schematic design showing condensation of anti-RhoC siRNA molecules by star-shaped pH sensitive polymers forming smart particles, which are internalized by endocytosis. In the endosome, acid-labile hydrazone linkages are hydrolyzed by the acidic pH, which release the P(HMA-co-DMAEMA-co-TMAEMA) grafts that rupture the endosomal membrane and release the loaded siRNA cargo into the cytoplasm. In the cytoplasm, anti-RhoC siRNA binds to RhoC mRNA triggering its degradation while particle debris is eliminated by exocytosis. Reproduced with permission from Kaushal *et al.* [149].

that excellent delivery of the drug can be obtained by combining both compounds.

Viral protein nanoparticles (VNPs) are another novel strategy for using non-polymeric nanocarriers as a drug-delivery system due to their excellent ability to mimic the natural process by which viruses penetrate cell membranes, with the added advantage that these nanoparticles are biocompatible and biodegradable. VNPs based on plant viruses and bacteriophages are excellent candidates as these viral proteins are not able to infect mammalian cells [153-155].

The use of one of these plant viruses for drug delivery has been evaluated by Aljabali *et al.*, who showed the ability of *Cowpea mosaic virus* (CPMV) to covalently conjugate with DOX in two ways, namely via a stable amide bond (CPMV-DOX) and a labile disulfide bridge (CPMV-SS-DOX). *In*

*in vitro* analyses in the HeLa cell line revealed that, at low dosage, CPMV-DOX is more effective for cell killing than free DOX because of its concentration-independent and time-delayed release. CPMV-DOX targets the endolysosomal compartment of HeLa cells and reduces the side effects of free DOX, such as the cardiotoxicity mentioned previously. The results for CPMV-SS-DOX revealed that this system exhibits a similar behaviour to DOX, possibly due to a more rapid dissociation of the disulfide-bridge, and therefore DOX delivery, prior to cellular uptake.

A further application of virus plant nanoparticles has been proposed by Esfandiari *et al.* Thus, a nanoparticle comprising potato virus X was conjugated with herceptin, a monoclonal anti-Her2/neu antibody that is used as a chemotherapeutic agent in breast cancer, via a chemical linker. *In vitro* flow cytometric studies in the SK-BR-3 and SK-OV-3 cell lines showed that, at different doses, PVX-Her2 showed a greater apoptotic effect than the same doses of free herceptin [156].

Metal organic frameworks (MOFs) comprise a combination of metals connected to organic molecules via a linker. These systems are attracting increasing interest in biomedical applications due to their excellent ability to encapsulate and protect the drug release, thus making them good systems for drug delivery [157]. In order to demonstrate the controlled delivery of drugs by such systems, Adhikari *et al.* designed a pH-responsive zeolitic imidazole framework (ZIF) containing zinc as the metal component and imidazole or another derivative as organic linker [158] with aim of encapsulating DOX within two different ZIFs. The resulting polymers showed an enormous difference in terms of their pH-responsiveness, with ZIF-8 demonstrating an efficient response capacity at acidic pH, releasing DOX in the required manner. However, in the presence of liposomes and micelles, both ZIFs exhibited a high release rate, especially ZIF-8, probably due to the destabilization of their respective structures. The authors of this study demonstrated that drug release can be controlled without difficulty using different ZIFs and, optionally, liposomes [159].

## CONCLUSION

ELRs have been extensively used for drug delivery in a variety of different forms, including soluble macromolecular carriers, self-assembled nanoparticles, cross-linked microparticles, thermally coacervated depots or crosslinked hydrogels. Targeted drug delivery using soluble macromolecular carriers encompasses different constructs, such as ELR-drug recombinant fusion constructs, ELR-drug systems chemically bioconjugated in their monomeric and soluble form, and drug encapsulation by nanoparticle-forming ELRs. These constructs allow controlled release at the target site, thereby suggesting the potential of elastin-like recombinamers as safe drug carriers for use in the treatment of different diseases.

The reversible phase transition that ELRs undergo in response to changes in temperature allows the formation of structure-dependent architectures, such as aggregates or well-defined nanoparticles, and the use of such systems in the biomedical field, especially for drug-delivery purposes and even for direct cellular uptake. Particularly interesting

examples of this can be found in cancer treatment, where the use of local mild hyperthermia and enhanced permeability retention has resulted in satisfactory treatments targeted directly at the tumour site.

The direct application of ELRs by injection as soluble monomers at the site of action to form depots extends drug exposure time while minimizing systemic side effects. This also paves the way to advanced medical applications, such as in tissue regeneration, neuroinflammation, cancer and diabetes. In addition, the use of ELR-based hydrogel systems that act as both a support and reservoir in which drugs can be stored results in a non-fluctuating and sustained drug release, thereby reducing side effects and increasing drug retention at the site of action with respect to the administration of free drugs.

## CONFLICT OF INTEREST

The authors declare no competing financial interest.

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