Elastin-like recombinamers: Biosynthetic strategies and biotechnological applications

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The past few decades have witnessed the development of novel naturally inspired biomimetic materials, such as polysaccharides and proteins. Likewise, the seemingly exponential evolution of genetic-engineering techniques and modern biotechnology has led to the emergence of advanced protein-based materials with multifunctional properties. This approach allows extraordinary control over the architecture of the polymer, and therefore, monodispersity, controlled physicochemical properties, and high sequence complexity that would otherwise be impossible to attain. Elastin-like recombinamers (ELRs) are emerging as some of the most prolific of these protein-based biopolymers. Indeed, their inherent properties, such as biocompatibility, smart nature, and mechanical qualities, make these recombinant polymers suitable for use in numerous biomedical and nanotechnology applications, such as tissue engineering, "smart" nanodevices, drug delivery, and protein purification. Herein, we present recent progress in the biotechnological applications of ELRs and the most important genetic engineering-based strategies used in their biosynthesis.

Keywords: Elastin-like recombinamers · Inverse temperature transition · Protein purification · Self-assembly · Stimuli-responsive materials

1 Introduction

Biological materials display extraordinary and amazing properties as a result of thousands of years of evolution during which natural selection has allowed only the best systems to survive. An understanding of how these biological materials are organized and display their functions is therefore required to create artificial materials and systems that mimic natural ones [1]. Over and above this, however, our aim should not only be to copy natural systems, but also to improve on them by creating bio-inspired compounds with enhanced properties that are not found in nature [2–4].

Natural biopolymers are an excellent example of the relationship that exists between sequence and the functions displayed. Of all biopolymers, this review focuses on describing elastin-like recombinamers (ELRs), bioproduction, and practical biotechnological applications.

The protein elastin is one of the most important constituents of the extracellular matrix. Although its main role is to provide elasticity to tissues [5], its biological function is not restricted to this task, since it plays an active role in modulating cell behavior and promoting tissue repair [6]. The primary sequence of elastin has regions governed by repeat motifs, such as VPGG, VPGVG, APGVG, and VGVAPG, the structure of which allows them to undergo high deformation without breaking and to return to their original conformation once the stress disappears [7]. Furthermore, this process has the peculiarity of occurring with no loss of energy. As a result of this energetic mechanism, the

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Abbreviations: ELR, elastin-like recombinamer; **HAP**, hydroxyapatite; **ITC**, inverse transition cycling; **ITT**, inverse temperature transition; **OEPCR**, overlap elongation polymerase chain reaction; **OERCA**, overlap-extension rolling circle amplification; **PRe-RDL**, recursive directional ligation by plasmid reconstruction; **RDL**, recursive directional ligation; **scFv**, single-chain variable fragment; T_t, transition temperature

resulting elastic fibers are able to undergo more than one billion relaxation–stretching cycles without suffering damage.

Elastin-like polymers (ELPs) are artificial polypeptides, the sequence of which mimics the repeat motifs found in natural elastin [7]. The primary sequence of the ELP is commonly governed by *n* repeats of the (VPGXG) motif, in which X is any amino acid except L-proline. The maturation of recombinant DNA technologies has allowed these protein-based materials to be synthesized in high yields, while retaining precise control over chain complexity, length, stoichiometry, and monodispersity [2, 8]. Indeed, a new term, namely, ELRs [9], for which "recombinamers" emphasizes the fact that these macromolecules have both an oligomeric and recombinant nature, has been created to evoke all these properties. Genetic engineering allows us to create advanced designs able to exhibit functions of particular technological significance not present in living organisms. As a result, the increasing availability of recombinant forms of elastin has led to the formation of a broad range of biomaterials and composites [3, 10, 11]. Apart from specific properties related to a particular design, ELRs also benefit from the inherent properties of elastin, in particular, biocompatibility, smart nature, and mechanical properties.

The mechanical performance of ELRs is accompanied by an extraordinary biocompatibility, since the host organism's immune system is unable to distinguish between endogenous elastin and an ELR when the biopolymer is based on the most common motifs present in the natural protein [12–16].

Moreover, ELRs show a reversible phase transition in response to temperature. Thus, in aqueous solution, and below a specific temperature known as the transition temperature (T_t), the polymer chain remains hydrated and surrounded by clathrate water structures. However, above T_t, clathrate water begins to get excited and its high level organization finally disappears [17]. The ELR then folds hydrophobically and assembles to form a separate phase. In this folded state, the chains adopt a regular, dynamic, nonrandom structure identified as a β spiral. The overall process has been termed the inverse temperature transition (ITT) [1] .

Numerous parameters, such as polymer concentration [18], amino acid composition [19], pH [20], and ionic strength [17], have to be taken into consideration when defining a specific T_t. In light of this, ELRs can be considered as smart polymers, since they are able to respond to several external stimuli, thereby sensing their microenvironment and undergoing changes in response to it.

As a result of the inherent function displayed by natural elastin in the organism, namely, to provide appropriate mechanical properties to tissues, tissue engineering was the first area in which the application of these polymers was studied. An enormous amount of scientific research has been undertaken in this area and the biomedical significance of the resulting applications has been broadly reviewed [2, 9, 10, 21–23]; therefore, it is not examined again herein. In contrast, a wide range of new applications, including protein purification, environmental restoration, drug delivery, stimuli-responsive materials, and surface engineering, have been developed since ELR-based tissue engineering became more widespread. The most common strategies used in ELR production, together with the biotechnological applications of these polymers, are covered in more detail below.

2 Biotechnological strategies for ELR production

2.1 Synthetic recombinamer genes: Design and construction

ELRs are progressive materials with physical, mechanical, and functional attributes derived from a combination of newly developed and natural protein elements, thus the sequences are artificial and the genes must be designed and synthesized de novo. The only methodological approach that currently allows repetitive protein polymers to be manufactured involves genetic engineering. The design and production of stable synthetic genes composed of a long codifying sequence of several small, highly repetitive artificial fragments, and subsequent expression in heterologous systems, brings with it its own problems. These problems are related to the absence of such monotonous DNA and protein sequences in nature, because they are subjected to a very high mutation rate, and the limitations of the biosynthetic machinery of the expression host. To facilitate the biosynthesis of an ELR codifying region, the biomolecular engineer has to choose the sequence by taking into account the mRNA structure and the most preferred codons for the expression host (usually *Escherichia coli*) [24], thereby searching for an equilibrium that avoids the collapse of the bacterial translational system as a result of the manufacture of such repetitive polypeptides. Finally, a high recombination frequency should be avoided when the exoge-

nous DNA contains multiple repeated DNA sequences [25].

2.2 Monomeric gene synthesis

Several biosynthetic strategies for ELR-encoding genes involve the construction of a monomeric DNA segment, or "monomeric gene", encoding a specific polymer sequence that is subsequently joined to generate multimerized genes expressing the whole recombinamer. The ends of the monomeric gene often contain specific endonuclease restriction sites, the termini of which are not palindromic when cleaved, thus leading to unidirectional "head-to-tail" ligation. Recently, the use of type IIS restriction endonucleases, which recognize asymmetric base sequences outside their cleavage site, has resulted in "seamless cloning" [26–28] and guarantees unidirectional ligation and avoids the insertion of unwanted nucleotides at the ligation joints, thereby avoiding extraneous amino acid residues [29].

Monomeric genes have also been synthesized by acellular approaches, such as the annealing of two fully complementary oligonucleotides [30, 31], the extension of two synthetic primers that are complementary only on their 3′ ends by PCR [32], the retrotranscription of two oligonucleotides that are complementary in one region [33], or by taking advantage of cellular DNA control and repair systems to obtain large amounts of high fidelity monomeric genes [34]. Although in vivo synthesis based on classical genetic engineering methods is laborious and slow, it nevertheless allows large amounts of monomeric gene with the correct sequence to be obtained in a controlled manner, thereby facilitating the combination of several monomeric genes to achieve more sophisticated and accurate gene assembly for the construction of block copolymers.

2.3 Methods for gene oligomerization

The synthesis of longer genes encoding repetitive protein-based recombinamers can be achieved by using random-oligomerization or controlled-multimerization methods (Table 1).

Table 1. Methods employed for ELR gene synthesis

Method[a)]	Procedure	Advantages	Disadvantages	Refs.
Concatemerization	Random head-to-tail ligation of monomeric genes	• Rapid: single-step synthesis of repetitive gene library • High sequence fidelity with recombinant genes	• Uncontrolled number and order of the gene fragments • Useful only for homopolymers • Low sequence fidelity with synthetic genes • Simultaneous formation of circular multimers as byproducts	[8, 26, 30, 31] [28, 35] [20, 36, 37]
OEPCR	Single-step synthetic process, involving overlap elongation PCR amplification	• Rapid: single-step synthesis of repetitive gene library • Productive: high amount of multimeric gene is obtained	• Uncontrolled number and order of the gene fragments • Increased error rate in gene sequences • Nonspecific priming and mismatch pairing	[38, 40]
OERCA	Combination of overlap elongation PCR and rolling-circle amplification techniques.	• Rapid: single-step synthesis of repetitive gene library • Control of the size of the library: rapidly achieve different pool size ranges of oligomerized genes	• Uncontrolled number and order of the gene fragments • Lower fidelity than cloning methods	[41]
RDL	Step-by-step directional recursive addition of monomeric genes	• Control of the number, order and succession of oligomerization • Construction of block copolymers	• Plasmid self-ligation • Insert circularization • Time-consuming method	[19, 36, 42, 46]
PRe-RDL	Step-by-step directional gene elongation by plasmid reconstruction	• Control of the number, order, and succession of oligomerization • Construction of block copolymers • High efficiency in cloning steps	• Time-consuming method • Wide set of restriction enzymes required	[43]

a) Abbreviations: OEPCR, overlap elongation polymerase chain reaction; OERCA, overlap-extension rolling circle amplification; RDL, recursive directional ligation; PRe-RDL, recursive directional ligation by plasmid reconstruction

2.3.1 *Random oligomerization*

Concatemerization is one of the most widely used methods for the construction of gene libraries containing different multimeric copies of the starting gene (Fig. 1A). As its name suggests, this method is based on "concatenation"; a random unidirectional ligation of DNA fragments that produces linear oligomers with a defined distribution and discrete lengths in a single cloning cycle [8, 30, 35]. Although this technique offers a powerful approach to the production of multimerized genes, it has several drawbacks, including an inability to control the order or number of repetitions and the presence of circular multimers of various lengths as byproducts [36, 37].

Another alternative and rapid approach, known as OEPCR, allows various repetitive DNA chain lengths to be obtained from short DNA oligonucleotides that act as primers and template simultaneously (Fig. 1B) [38]. The 3′ termini of the oligonucleotides are complementary and can hybridize to form a duplex with a 5′ overhang that DNA polymerase fills in to produce DNA duplexes. This process is repeated in subsequent cycles in which the PCR products are extended to longer sizes. Although this approach is faster and more productive, it nevertheless has the drawback of low specificity. Indeed, the high GC content of ELR-encoding monomeric genes and their repetitiveness, the higher error rate of DNA polymerases, and the frequency of "non-specific priming", or "mismatch pairing", decreases the fidelity with respect to the products obtained when using standard recombinant DNA techniques [39]. A more recent modification of this method, which is based on serial PCR amplifications using three different oligonucleotides, is known as non-template PCR. Thus, in addition to the two usual 5′ and 3′ primers, this method includes a third "elongation primer", the codifying sequence of which has complementary sites at both ends, thus allowing it to partially anneal with itself or with one of the other primers, and therefore, be used in different sequential PCRs with one of the other partners to generate multiple repeats of the monomeric gene [40].

Chilkoti and co-workers recently developed a different random oligomerization method to rapidly achieve long repetitive genes [41]. This method,

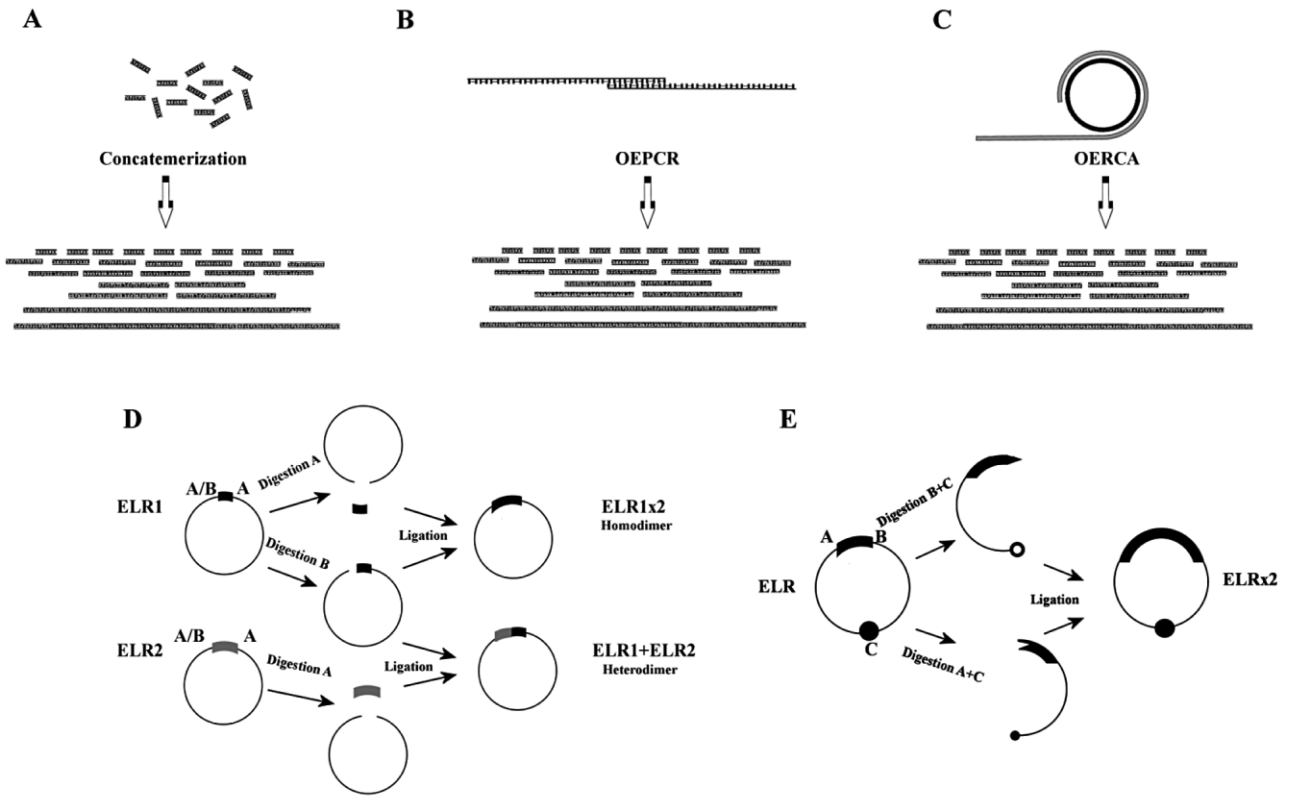

Figure 1. Random or controlled approaches to ELR gene oligomerization. Schematic representation of the oligomerization techniques utilized to obtain larger genes from short repeat motifs. Concatemerization (**A**), OEPCR (**B**), and OERCA (**C**) are faster, random synthetic approaches that do not allow control of the size or insertion order. Such control can instead be obtained by RDL (**D**) and PRe-RDL (**E**), for which single, controlled fragment addition is obtained in each cloning cycle.

known as OERCA, allows the highly parallel construction of ELR-encoding genes by combining RCA with OEPCR techniques (Fig. 1C). They described gene oligomerization that provides a library of repetitive genes with high sequence fidelity and showed how it could be performed in simple PCR steps. In this case, the template is a circularized single-stranded DNA (ssDNA) that encodes the monomeric gene duplicated in vitro to create complementary DNA fragments. Subsequent PCR amplifications, using forward and reverse primers, initiate overlap extension reactions that produce long, repetitive DNA oligomers. Moreover, variations in the reaction conditions (primer-to-template ratio or thermal cycling protocol) alter the size distribution, thereby resulting in the rapid production of different size ranges of oligomerized genes [41].

2.3.2 Sequential methods

Although random multimerization techniques offer a powerful and robust approach to the synthesis of multimerized genes, they all present some limitations, especially with regards to the inability to control the order or number of repetitions in the concatenation process. If the polymer's composition is more complex and absolute control over its sequence is essential, the gene sequence must therefore be constructed by a step-by-step directional approach.

Block copolymer architectures, for example, require the use of a strategy in which the biomolecular engineer is able to strictly control all characteristics that play central roles in the formation of supramolecular structures, such as the size, monomer addition sequence, and distribution of the individual blocks.

One of the most widely employed controlled multimerization methods is known as RDL and involves oligomerization of the DNA fragment in a succession of single and uniform steps (Fig. 1D). Each of these steps grows the polymer gene by one block length of the monomer [42]. The vector containing the monomer is used as both insert donor and receptor to duplicate the gene by seamless cloning. Additional RDL cycles proceed identically, using products from previous rounds as starting materials, until a gene of the desired length or architecture is obtained [19, 42].

In a more recent approach, Chilkoti and coworkers [43] reported a new adaptation of RDL, namely, PRe-RDL, in which they dimerized two halves of a vector containing a copy of the ELR gene to reconstruct a functional plasmid (Fig. 1E). This new technique solves two of the major drawbacks of its predecessor, namely, self-ligation of the plasmid and the low efficiency caused by nonproductive circularized forms of the gene fragment during ligation [43].

2.4 Heterologous expression systems

Due to its many advantages, including the fact that it is well characterized, its genome sequence is known, many of its biological processes and metabolic pathways are understood, and there are many readily available genetic tools for its manipulation, *E. coli* is the most widely used heterologous expression system for ELR production.

These benefits of using *E. coli* are reflected in the countless examples of different ELRs that have been produced using this bacterium, which range from free ELRs to chimeric constructs in which the ELR acts as a tool for downstream processing. Typical ELR yields of approximately 300 mg/L have been reached; however, obtaining such yields is not a trivial task because many aspects have to be taken into account [44, 45]. Thus, as discussed previously, special care is required when designing ELR genes because of their repetitive nature. Moreover, this aspect can also have detrimental effects in terms of yield because specific amino acids, such as glycine, L-proline, or L-valine, are not present in sufficient quantity to achieve successful protein translation. Indeed, in this respect, it has been reported that the expression levels of an ELR can be greatly improved by adding specific amino acids [46, 47]. Another approach to avoid yield problems is the construction of a metabolically engineered *E. coli* typified by an increase in the aminoacyl-tRNA pool of such specific amino acids [48].

Special attention must also be paid to the presence of endotoxins [49] in the final product. In light of the biomedical applications of ELRs, it is therefore vital to perform a specific protocol to remove such compounds [16, 50] and to determine their levels before application in vivo. Such protocols take advantage of the smart nature of ELRs by including heating–cooling cycles combined with treatment with sodium hydroxide.

Because ELRs are recovered from inside cells in the *E. coli* expression system, an alternative expression system based on *Pichia pastoris* as a host organism has been studied to eliminate the cell-disruption step during the purification process [51, 52]. *P. pastoris* secretes the ELR, thereby allowing ELR purification directly from the culture medium with yields of 255 mg/L of cell-free medium [52]. Furthermore, yeasts do not produce endotoxins, thereby further simplifying the downstream processing of the ELR. Besides allowing protein secretion, this lower eukaryotic system contains the ap-

propriate intracellular machinery to allow post-translational modifications and folding assistance, which may be relevant in the case of chimeric constructs.

Higher eukaryotic systems, especially plants, have also been used as ELR production factories. Indeed, since the polypeptide (VPGVG)121 was first purified from transgenic tobacco leaves as far back as 1995 [53], the range of ELRs produced in plants has expanded enormously [54]. For instance, tobacco seeds have been chosen for the expression of functional scFv linked to an ELR and to the endoplasmic reticulum (ER) through a KDEL sequence [55]. Seeds are particularly practical organs for molecular farming due to their ability to maintain protein quality under ambient conditions during long-term storage [56]. A further reported advantage of scFv–ELR fusion constructs produced in plants is the enhanced expression yield provided by ELRs [57, 58], for example, a 40-fold increase in scFv accumulation of the ELPylated form was reported [55]. ER retention in tobacco leaf cells was also chosen as the intracellular destination for the ELPylated chimeric protein gp130 (a natural glycoprotein able to block IL-6 signaling) [59] and for the TBAg-ELR fusion construct (TBAg is a bipartite protein consisting of Ag85B and ESSAT-6 antigens from *Mycobacterium tuberculosis*), which shows promising vaccine potential [60]. Both approaches show the potentially great impact that diagnostic and therapeutic proteins produced in transgenic plants can exert on human healthcare.

3 Biotechnological applications of ELRs

3.1 Chimeric constructs with ELRs

Although chimeric constructs with an ELR were initially devised as a strategy to achieve successful protein purification [61], the role of the ELR in protein construction is not limited to this task. Indeed, several other beneficial collateral effects make an ELR a powerful tag with widespread use in numerous practical applications.

3.1.1 Protein purification by ELRs

Although chromatography is the standard methodology for the successful purification of recombinant proteins, its many disadvantages, including the need for specialized equipment and difficulties scaling up, have driven the search for novel purification methods that overcome these problems. One such approach exploits the inherent properties of ELRs.

As mentioned in the Introduction, one of the most astonishing properties of ELRs is their thermosensitive and smart behavior. ELR-based protein purification takes advantage of the fact that these properties are maintained when produced as recombinant fusions along with exogenous proteins [61]. This observation resulted in a scientific breakthrough in the field of protein purification because ELRs are able to work as environmentally sensitive tags for the purification of recombinant proteins, thereby providing an interesting alternative to chromatography. This new technique is called inverse transition cycling (ITC) and consists of several sequential and repeated steps of heating, centrifugation, cooling, and solubilization (Fig. 2) [61].

Although a standard protocol has been established to purify chimeric proteins by ITC [61], some optimization is required for each target protein [62, 63]. This optimization involves determination of ITC parameters (such as temperature [64], salt concentration [17, 64], and pH [65]) as well as optimization of the fusion protein design [62]. As far as the ELPylated construct is concerned, the guest residue composition of the ELR [65, 66], its length [28, 61, 67], and fusion order [68] must also be evaluated.

Since the thermoresponsiveness of the ELR depends, amongst other parameters, on the polymer concentration, several problems have been encountered with the ITC approach when applied to proteins expressed at ultra-low levels. Thus, if the ELR is not present above a minimal concentration, suitable aggregates are not properly formed and efficient recovery is not possible [25]. Nevertheless, the ITC approach has evolved to circumvent this problem by the addition of free ELR, which acts as a co-aggregant [25, 69].

Conventional ELR tagging suffers from two additional drawbacks. The first of these is related to cleavage of the ELR tag, which can increase the cost of large-scale purification due to the use of enzymes, which are usually expensive. Moreover, although the ELR can easily be segregated from its target protein by triggering post-cleavage thermal aggregation (Fig. 2L), the specific protease must also be removed from the final product, which can often prove difficult. Further modifications of conventional ELR tagging have, however, been developed to overcome these problems.

The first such modification, known as ELP-mediated affinity capture (EMAC) [70], makes use of an ELPylated construct in which the target protein is an antibody-binding domain (protein G, L or LG). Once the interaction with the antibody has occurred, transition of the ELR, which leads to recov-

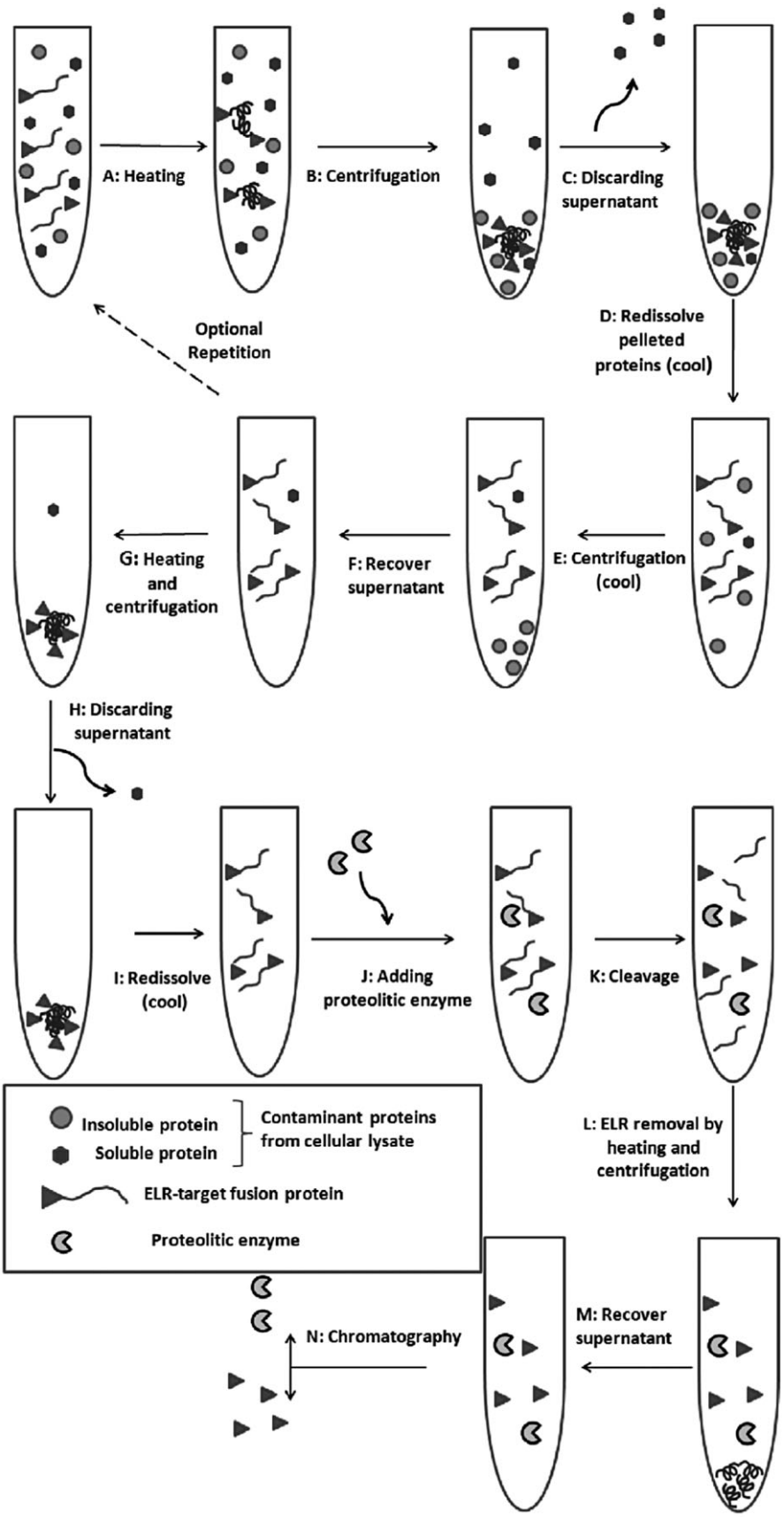

Figure 2. Diagram of a typical protein purification protocol based on ITC. ELR ITT can be triggered by heat, the addition of NaCl, or changes in pH (**A**). As a consequence, the chimeric construct can easily be recovered by centrifugation (**B**, **C**). However, insoluble proteins (circles) and some soluble contaminants (hexagons) can co-localize with the polymer in the pelleted fraction. However, they can be redissolved in cool water (**D**), and further centrifugation under cool conditions separates the insoluble host proteins from the soluble fraction. The supernatant is again subjected to a heating cycle (**G**, **H**), soluble contaminants are discarded, and the ELR is redissolved in cool buffer (**I**). Purification of the ELR fusion protein may therefore need multiple rounds of ITC and some optimization. Furthermore, when necessary, the ELR can be removed from the target protein after specific protease cleavage (**J**, **K**), and by a simple additional ITC step (**L**, **M**). In this case, a post-purification step may be required to separate the target protein from the proteolytic enzyme (**N**). Alternatively, intein technology can be used to avoid these steps.

ery of the antibody, is triggered [70]. The main benefits of this approach are (1) its universality, since a single ELPylated construct is able to purify different antibodies; (2) the absence of enzymatic cleavage during the purification process; and (3) the consequent reduction in the cost of target protein production.

ELR fusion constructs can also be developed by using the intein technique [71]. The intein peptide is able to trigger inducible self-cleavage under mild conditions (room temperature and neutral pH). Several tripartite fusion constructs, containing an ELR, the intein sequence, and a target protein, have been reported, for example, the antimicrobial peptides CM4 and HβD4 have been fused downstream of ELR-intein and expressed in *E. coli* [72].

Although the initial establishment of a rational method for designing ELR fusion constructs was mainly carried out in *E. coli*, an increasing number of ELPylated proteins have been successfully purified in plants, as noted above [54, 55, 57, 58, 60]. ELPylated proteins in general, especially those expressed in plants, profit from simpler downstream processing and also from further functions of the ELR tags, as discussed in detail below.

3.1.2 Advanced functions of ELR tags in chimeric constructs

ELR tags are also believed to assist the folding of target proteins by sterically hindering the aggregation of folding intermediates of the fusion protein. Moreover, steric effects can be useful in avoiding protease attack. The ELR could therefore be considered as a shield that enhances the stability of the target proteins and protects them from proteolytic degradation.

The next scientific challenge of these steric effects is to determine whether the ELR can mask and interfere with either the biological activity or the necessary post-translational modifications of some target proteins. In contrast, countless examples of active and appropriately glycosylated ELPylated proteins have been reported [54, 62].

Further contributions of ELR tags, apart from protective effects, were described. Indeed, such tags are emerging as promising carriers for pharmaceutically active proteins. One of the drawbacks of many potential pharmaceutical compounds is their short serum half-life; however, it was reported that it could be lengthened by ELPylation. One illustrative example involved the ELPylation of a single-domain monoclonal antibody that recognized TNF (TNF-VhH). This fusion construct possessed a substantially longer (24-fold) serum half-life than that of the naked antibody fragment [57].

By taking advantage of the increased drug half-life of proteins fused to ELR, PhaseBio is developing ELPylated drugs to treat several diseases. The company's most promising candidate is currently Glymera™ (in which glucagon-like peptide-1 (GLP-1) analogue is the target protein), which is undergoing clinical testing for the treatment of type 2 diabetes and obesity.

With regards to scFv, an anti-fluorescein scFv has been developed that is constructed by fusing variable light (VL) and variable heavy (VH) domains with a short ELR (sELR), thus reconstituting a single binding site. It was subsequently demonstrated that the binding affinity of this construct decreased as the temperature increased due to increased molecular ordering known for ELRs. This ELR contraction creates stress responsible for disrupting the interaction between the two variable domains, thereby facilitating ligand release [73].

The use of ELRs as tags is not restricted to biomedical applications because they are also useful in other fields, such as the textile industry. Thus, the recent ELR engineering of subtilisin E represented a breakthrough in wool finishing because the ELPylated enzyme was only able to hydrolyze the cuticle layer of wool thanks to the increased molecular weight provided by the polymer. This prevented subtilisin E from diffusing into the wool cortex, thereby avoiding undesirable effects on the fiber structure [74].

Beside protein purification, ELRs are also useful for the purification or removal of nonprotein compounds. For example, plasmid DNA has been purified by using an ELR chimeric construct fused to a bacterial metalloregulatory protein that binds to a specific DNA sequence present in the plasmid [75].

ELRs have also been employed as environmentally benign chelating agents by fusing a polyhistidine domain and an ELR into a single polypeptide [31, 76, 77] or into copolymer ELRs to form hydrogels [78], which were then used for the treatment of heavy-metal-contaminated wastewater streams. Likewise, an ELR containing synthetic phytochelatin, which contains a strong and highly selective metal-binding domain, has been biosynthesized for cadmium extraction from contaminated soil. Cadmium was removed from both exchangeable and oxidizable fractions [79].

Additionally, a bifunctional fusion protein composed of organophosphorus hydrolase and an ELR was synthesized for the detoxification of organophosphorus compounds in pesticide residues [80]. These examples show us the versatility of ELRs for a practical biotechnology application concerning

environmental pollution where the contaminants have diverse chemical compositions.

3.2 Various stimuli-responsive formulations based on ELRs

The modular nature of the sequence of ELRs allows them to be rationally designed for use in specific nanotechnology or biomedical applications, such as nanoswitches, smart surfaces, diagnostic biosensors, and drug delivery. Thus, ELRs employed as drug carriers [35] have been designed to take full advantage of several inherent properties, such as biocompatibility, thermoresponsive ability to self-assemble into definite and monodisperse macromolecular structures [81, 82], molecular-weight control as a key parameter in pharmacokinetics [83, 84], molecular architecture control to create amphiphilic block copolymers to modulate particle size [19, 85], and the incorporation of bioactive motifs (targeting peptides [86], antibodies [57, 73], or receptor ligands [87]) that specifically recognize and interact with their own target into the ELR sequence. Furthermore, several ELRs have been designed to self-assemble in response to external stimuli, such as the local application of heat, ultrasound, or light, or to the local microenvironment, such as extracellular pH or protease expression [88]. In cancer drug delivery, "thermal targeting" obtained upon fine-tuning of T_t increased local ELR–drug complex accumulation in hyperthermic tumors, thereby limiting undesirable systemic toxicity [89], whereas "active targeting" by the inclusion of cell-penetrating and therapeutic peptides in ELRs enhanced their intracellular delivery [90].

The properties of ELRs have resulted in an expansion of their use as stimuli-responsive nanostructured materials. Thus, Reguera et al. [20] reported the formation of self-assembled, equally spaced nanopores on the surface of a recombinamer that exhibited stimuli-responsive behavior in response to both pH and temperature (Fig. 3). ELRs were also employed as a trigger for blocking and opening the cavity of staphylococcal a-hemolysin (aHL) pores [91]. Thermoresponsive pores have also found applications in drug release [92] or the permeabilization of mammalian cells [91].

In the field of bionanotechnology, stimuli-responsive ELRs have been used to produce smart surfaces for bioanalytical applications by covalent micropatterning onto a glass surface, such as the so-called thermodynamically reversible addressing of proteins (TRAP), and to modulate ligand-binding activation by ELR ITT at a solid–liquid interface [93, 94]. Bioactive surfaces that can modulate cell response were produced by layer-by-layer deposition of ELR–polyelectrolytes. A thin coating of a bioactive ELR onto chitosan surfaces by electrostatic self-assembly (ESA) determined the formation of nanoscale smart systems used to modulate cell adhesion and protein adsorption [95]. Two examples of ELRs able to bind to hydroxyapatite (HAP) or to nucleate mineralization were described recently. Wang et al. [96] reported that a

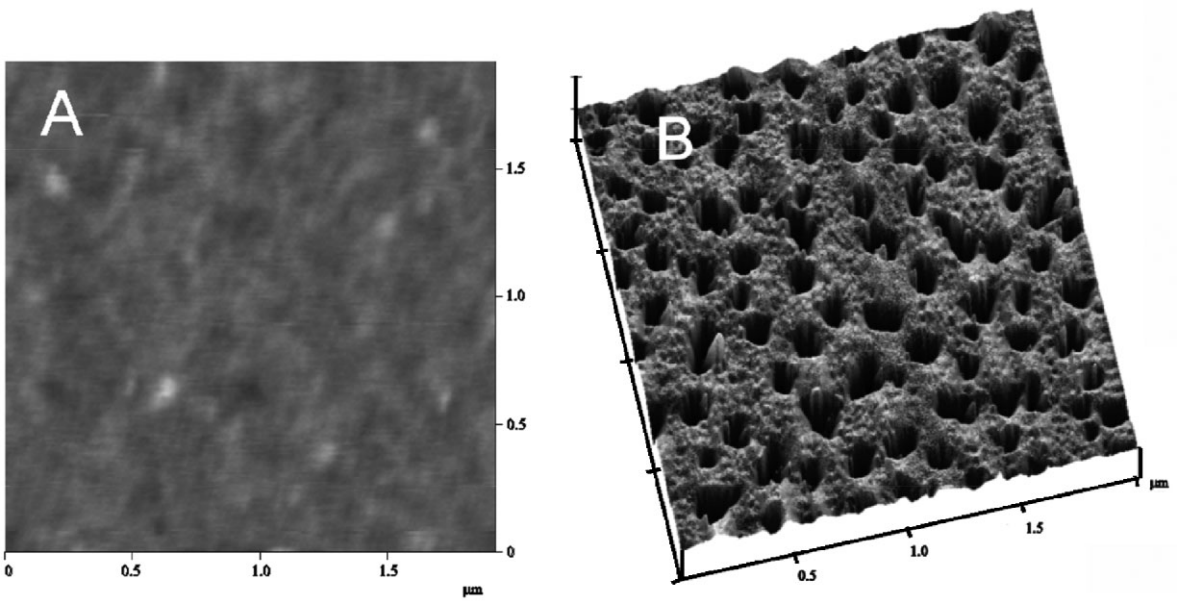

Figure 3. AFM images of the pH-responsive surface of ELRs. An aqueous solution of the ELR was deposited onto a hydrophobic Si substrate. Self-assembling nanopore formation requires control of the pH of the ELR solutions. Sample conditions: (**A**) 10 mg/mL in a 0.02 M aqueous solution of HCl (acid solution); (**B**) 10 mg/mL in a 0.02 M aqueous solution of NaOH (basic solution) [20].

mixture of ELRs containing hydroxyapatite (HAP)-binding octaglutamic acid motifs and HAP crystals in solution exhibited a sequence-specific ability to bind HAP and disperse HAP nanoparticles.

Prieto et al. [97] reported that a set of biofunctionalized ELRs spontaneously generated biomimetic hybrid materials that acted as crystallization additives for calcium phosphate deposition. In this study, the ELR backbones included a sequence that promoted the mineralization of calcium in simulated body fluid (SBF), namely, the SNA15 domain of the salivary protein statherin. The recombinamer architecture was a key parameter for efficiently controlled calcium phosphate nucleation and the successful formation of spherical HAP.

4 Concluding remarks

The great versatility of protein-based biomaterials, together with the excellent properties of natural elastin, has allowed the development of a new kind of biopolymers, known as ELPs. Furthermore, the general improvement in recombinant DNA technologies over the past few decades has stimulated exponential growth of the corresponding recombinant version, known as ELRs. The more recent development of advanced "multimerization" strategies based on novel enzymatic tools and the improvement in gene amplification techniques has provided us with alternative methods for the rapid development of new ELRs with an extremely high degree of both functional and structural complexity. Herein, we have summarized these new methods along with the relevant heterologous systems applied in their biosynthesis.

The milestone for this kind of biomaterial should be to bring the first ELR-based device to market. Research published on these biopolymers has proved that the technology is mature and the wide range of reported applications (cell culture, tissue engineering, drug delivery, protein purification, environmental detoxification, nanobiotechnology, etc.) opens up the possibility for the introduction of new patentable compositions. However, although biomedical uses are always the most attractive, they are also quite complicated to achieve. Biotechnological applications therefore seem to be more promising in the near future and small companies recently set up with interesting ELR products in the pipeline should show us their true importance.

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