

Chapter 2

Synthesis of Genetically Engineered Protein Polymers (Recombinamers) as an Example of Advanced Self-Assembled Smart Materials

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Abstract

In this chapter, we describe two methods for bio-producing recombinant repetitive polypeptide polymers for use in biomedical devices. These polymers, known as elastin-like recombinamers (ELRs), are derived from the repetition of selected amino acid domains of extracellular matrix proteins with the aim of recreating their mechanical and physiological features. The proteinaceous nature of ELRs allows us to make use of the natural biosynthetic machinery of heterologous hosts to express advanced and large polymers or “recombinamers.” Despite the essentially unlimited possibilities for designing recombinamers, the production of synthetic genes to encode them should allow us to overcome the difficulties surrounding bioproduction of these non-natural monotonous DNA and protein sequences. The aim of this work is to supply the biotechnologist with fine-tuning methods to biosynthesize advanced self-assembled smart materials.

Key words: Elastin-like recombinamers, Genetically engineered protein polymers, Tissue engineering biomaterials, Self-assembly

1. Introduction

The biosynthesis of peptide polymers using natural pathways allows monodisperse and extremely sophisticated polymers to be synthesized (1). Biomaterial properties such as composition, molecular weight, and monodispersity affect pharmacokinetics and cellular-transport phenomena, therefore complete sequence and architecture control allows such materials to be designed with an extremely high degree of both functional and structural complexity (2). This control is now possible by constructing synthetic genes, which can include any sequence that encodes functional peptide domains that confer

mechanical, structural, or bioactive properties and also distribute and collocate them with the required ratio and consecutiveness (3). The resulting biopolymers, or recombinamers, possess enormous potential as advanced materials for a variety of biomedical applications, such as cell harvesting, drug delivery, and tissue engineering in the formation of different kinds of 2D or 3D scaffolds (4).

The two elastin-like recombinamers (ELRs) described herein have been designed to accomplish two specific biomedical purposes: to act as advanced scaffolds for tissue engineering and as self-assembled drug-delivery biomaterials (5). The first ELR contains different building blocks, namely the elastomeric pentapeptide VPGIG, which confers the desired mechanical behavior and biocompatibility, and a variation of VPGIG that contains a L-lysine instead of an L-isoleucine for cross-linking purposes. The polymer also contains two bioactive domains for cell adhesion and specific biodegradation. Thus, the CS5 fibronectin domain includes the REDV tetrapeptide, which is specifically recognized by the integrin $\alpha 4 \beta 1$ (6) present in endothelial cells selectively bound to REDV-coated surfaces (7). The other functional block (ValGlyValAlaProGly (VGVAPG)₃) is a recurring hexapeptide derived from the human elastin exon 24-encoded product (8). This sequence has been introduced to drive enzymatic hydrolysis of the synthetic scaffold by the same physiological pathways as natural elastin during extracellular matrix (ECM) remodeling (8).

The use of block copolymers in nanobiotechnology has aroused a great deal of interest over the past few years as their self-assembly properties enable the creation of different structures on a nanometer scale in an easy and inexpensive manner using a *bottom-up* approach (9). Amphiphilic block copolymers contain well-defined blocks of compositionally dissimilar monomers that have significantly different polarities and interaction affinities for aqueous solutions (10). Thus, amphiphilic ELR-based block copolymers comprise an apolar, hydrophobic block (in the second example above we used the L-alanine containing pentapeptide (A-block)) and a polar, hydrophilic block, in this case the pH-sensitive L-glutamic acid containing block (E-block). This type of molecule is ideally suited for applications involving the energetic and structural control of materials and biological interfaces, including emulsifiers, delivery agents, dispersants, gelation agents, compatibilizers, and foamants (11). Self-assembled micellar nanoparticles have recently been successfully employed in tissue engineering for targeted drug-delivery applications (12). The intrinsic self-assembly behavior of these amphiphilic molecules allows the synthesis of scaffolds suitable for cell culture in a layer-by-layer manner.

The ELR genes described herein are obtained by different approaches: concatemerization (13) and iterative-recursive methods (14). These procedures allow monomer genes to be “polymerized” in a seamless and unidirectional manner. This type of seamless cloning involves the use of type IIS restriction endonucleases that

recognize asymmetric base sequences and cleave DNA outside their recognition site, thereby cleaving any DNA sequence that is at a defined distance from their recognition sequence. These endonucleases are ideal for a protein polymer biosynthesis approach as they guarantee unidirectional ligation and avoid the insertion of extraneous DNA at the ligation joints, thereby eliminating extraneous amino acid residues within the polymer sequence.

The purpose of this chapter is to describe different methods available for obtaining ELRs polymeric genes synthesis, or for any other proteinaceous modular polymers, their heterologous expression in a bacterial system and further purification (Fig. 1), and finally two examples of biomedical devices construction are based on these biomaterials.

2. Materials

2.1. Monomeric Gene Production

1. Monomeric ELRs and oligonucleotide primers (IBA Nucleic Acid Synthesis). Restriction sites are indicated in bold type. Overlapping sequences are underlined:

REDV peptidic monomer: (VPGIG)₂VPGKG (VPGIG)₂
EEIQIGHIPREDVDYHLYP (VPGIG)₂VPGKG (VPGIG)₂
(VGAPG)₃

- (a) *REDV For*:

5' **ACCACTCTTCAGTACCGGGCATCGGTG**
TTCCGGGCATTGGTGTGCCGGGCAAAGGTGTT
CCGGGCATTGGTGTGGCCGGCATTGGTGAAG
AAATCCAGATCGGGCATATCCCACGCGAGGA
TGTGGACTACCACCTGTATCCGGTGCC 3'

- (b) *REDV Rev*:

5' **ACCACTCTTCTTACACCCGGTGCAA**
CGCCACACCCGGGGCAACGCCACACCCGGC
GCAACGCCAGGGGCAACGCCACACCCGGC
GCAACGCCCACTTTGCCCGGAACACCA
ATGCCCGGCACACCAATGCCTGGCACCGGATAC
AGGTGGTAGTCC 3'

A-block peptidic monomer: (VPAVG)₂₀

- (c) *VPAVG for*:

5' **AACGCTCTTCAGTACCGGCCGTGGGCGTT**
CCAGCAGTGGGTGTTCCGGCTGTGGGCGTTC
CGGCGGTGGGTGTTCCGGCGGTGGGCGTTC
GGCTGTGGGTGTTCCGGCGGTGGGCGTTC
TGCGGTGGGTGTTCCAGCAGTGGGTGTTCTGC
CGT CGGGGTCCCGGCCGTC 3'

- (d) *VPAVG rev*:

5' **AACACTCTTCTTACGCCCACTGCCGG**
AACACCCACGGCTGGAACGCCACAGCCGGA

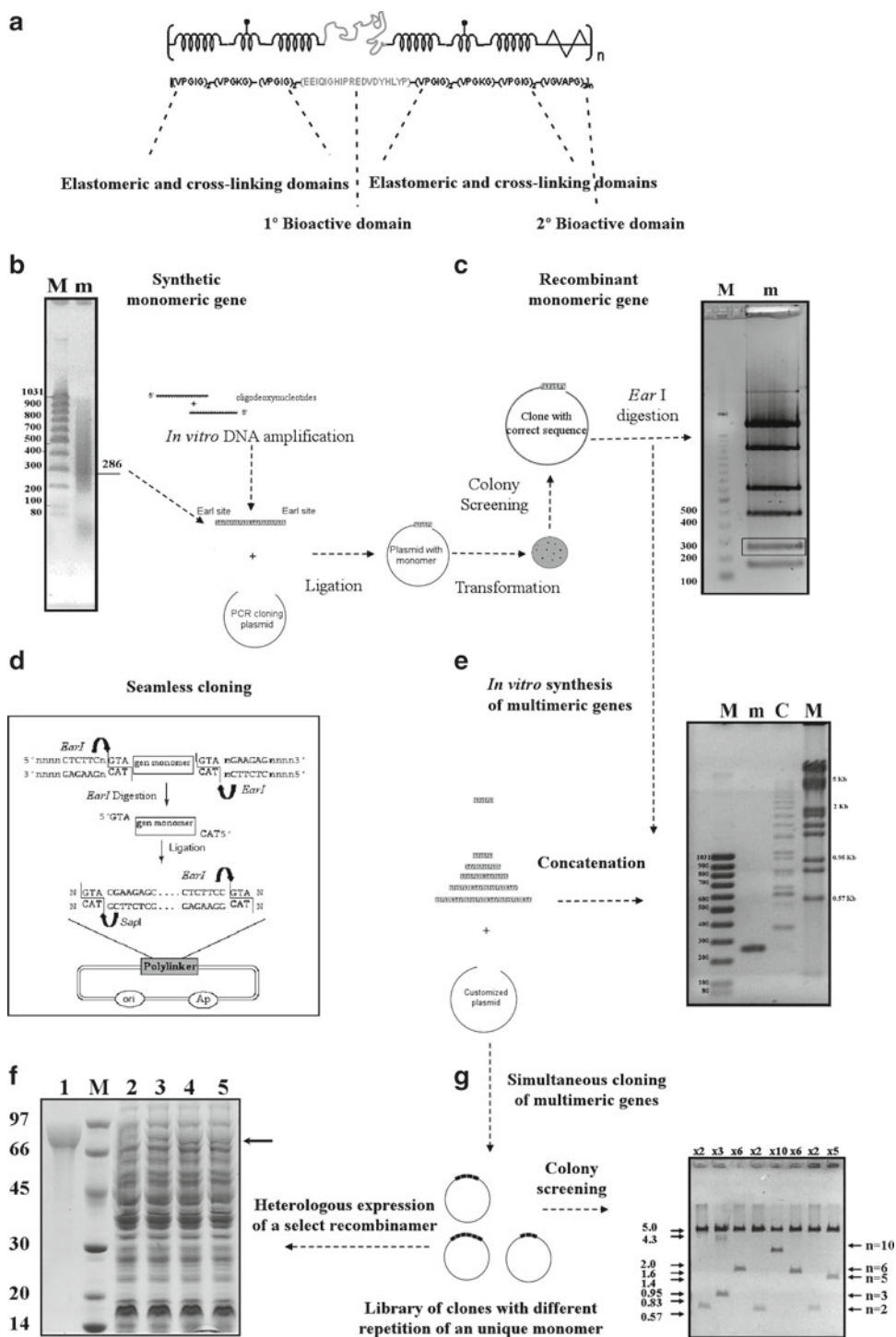


Fig. 1. Schematic representation of the concatemerization method for obtaining bioactive ELRs.

CACCCACCGCCGGAACGCCCACCGCCGGAACA
 CCCACCGCCGGAACGCCCACCGCCGGAACA
 CCCACCGCAGGAACGCCCCTGCTGGAACACC
GACGGCCGGGACCCCGACG 3'

E-block peptidic monomer: VPGVG VPGVG VPGEV
 VPGVG VPGVG

(e) *VPGEV for*:

5' AGTTAGGATCCCTCTTCAGTACCAGG
 TGTGTTGGTGTTCGGGTGTTGGCGTGCCGG
GCGAAGGCGTGCCG 3'

(f) *VPGEV rev*:

5' AGTTAGAATTCTCTTCCTTACCCCTA
 CACCCGGAACACCAACACCCGCGCACGCGCT
TCGCCCCGGCACG 3

2. dNTP mix (10 mM) (Eppendorf) (see Note 1).
3. Proof reading *Pfu* DNA polymerase and buffer (Stratagene).
4. Sterile nuclease-free water.
5. Blunt-Ended PCR Cloning Kit (GE Healthcare). Store at -20°C .
6. 2–3% Agarose gel: 2–3% (w/v) agarose MetaPhor (Cambrex) in 1× TAE buffer (see Note 2).
7. SYBR Green I Nucleic Acid Gel Stain (Invitrogene).

2.2. Plasmid Customization: Site-Directed Mutagenesis

1. pET-25b(+) Expression plasmid (Novagen).
2. Oligonucleotides for mutagenesis (Thermo Electron Corporation):
 pET-25b(+) *SapI* deletion
 (g) *Site 3153 for*: 5' CCGCATCAGGCGGTCTTCCGCTTCC 3'
 (h) *Site 3153 rev*: 5' GGAAGCGGAAGACCGCCTGATGCGG 3'
 pET-25b(+) Polylinker mutation. Both primers were purchased 5' phosphorylated.
 (i) *SitelinkerpETPhosF*: 5'GATATCGCTCTTCAGTATGAT
 CCGAATTTCGAGCTCCGTCGAC3'.
 (j) *SitelinkerpETPhosR*: 5'GACCAGCTCTTCCTACCGG
 CAGCAGGG3'.
3. Quick Change Site-directed Mutagenesis Kit (Stratagene).
4. pDrive Cloning Vector (Qiagen).
5. XL1-blue *E. coli* competent cells (Stratagene) (see Note 3).
6. 1% Agarose gel: 1% (w/v) agarose in 1× TAE buffer (40 mM Tris-acetate, 1 mM pH 8 EDTA (ethylenediaminetetraacetic acid)).
7. Molecular weight markers: PCR DNA ladder (New England Biolabs), 100 bp DNA ladder (New England Biolabs); 1,000 bp DNA ladder (Novagen).

2.3. Cloning and Purification of ELR Genes

1. Luria–Bertani medium (LB-amp): LB + 100 µg/mL ampicillin (Apollo) (see Note 4).
2. Luria–Bertani medium agar plates: LB-amp + 1.5% agar. Stable at 4°C up to 2 weeks.
3. Quantum Prep Plasmid Mini/Midiprep Kits (Biorad).
4. Restriction endonucleases and buffers, T4 DNA ligase and buffer, shrimp alkaline phosphatase (SAP) and buffer (Fermentas).
5. QIAquick Gel Extraction Kit (Qiagen).
6. Glycogen (Roche).

2.4. ELR Expression and Purification

1. BLR(DE3) *E. coli* expression strain (Novagen) (see Note 5).
2. IPTG (isopropyl-β-D-thiogalactopyranoside) (Apollo Scientific Ltd). Dissolve in water at 100 mM, filter-sterilize, and store at 4°C.
3. 1× TBS (Tris-Buffered Saline): 20 mM Tris-base pH 8, 140 mM NaCl (Sigma-Aldrich). Store at 4°C.
4. 1× SB (Sonication Buffer): 20 mM Tris-base, 1 mM pH 8 EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride) (Apollo Scientific Ltd). Store at 4°C.
5. Millex-VV 0.1 µm filters (Millipore).

2.5. Biomedical and Nanotechnological Applications of ELRs

1. REDV-ELR.
2. Sterile type I water.
3. Phosphate-buffered saline (abbreviated PBS).
4. *N,N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), hexamethylene diisocyanate (HDI), and acetone.
5. Circular cover glasses (Thermo Scientific).
6. Teflon molds.
7. E₅₀A₆₀ ELR copolymer.
8. Varian Cary 50 UV–vis spectrophotometer with a thermosta-tized sample chamber.
9. JEM2200FS Electronic Microscope (JEOL, Europe) GATAN magnification system (Gatan Inc, France), CCD camera US400, Digital Micrograph software.

3. Methods

3.1. Monomeric Gene Production

The artificial nature of ELRs means that it is necessary to design and synthesize their genes *de novo* in order to obtain them. Due to current methodological and technical limitations it is impossible to obtain a complete synthetic gene; therefore, the only possible approach involves genetic engineering. The repetitive structure of

recombinamers implies that a monomer DNA segment or “monomeric gene” encoding a specific polymer sequence can be created and subsequently seamlessly joined independently of the presence of restriction sites in the target DNA to generate multimerized genes that express the whole recombinamer.

3.1.1. Oligomer Design Guidelines

The ELR monomeric genes were synthesized by PCR amplification of oligonucleotides (Fig. 1b) which have to be designed to act simultaneously as primers and template. Their sequences should contain three functional regions (see Note 6). The 5' terminal contains the sequences necessary for seamless cloning and/or multimerization and comprises the type IIS restriction enzymes (*EarI* or *SapI*) recognition site (see Note 7) along with the sequence of protruding DNA that governs the unidirectional ligation and codifies for the first and last codons of the ORF (in this case GTA for L-valine). The sequence of the codifying region was chosen taking into account the mRNA structure and the most preferred codons for the heterologous expression system (usually *E. coli*) (15, 16), searching for an equilibrium that avoids collapse of the bacterial translational system due to fabrication of such repetitive polypeptides. The 3' terminal of every oligonucleotide couple is the complementary hybridization region that functions as primer. It is a codifying region that contains the most infrequent codons of the oligonucleotides in order to improve the specificity of the annealing zone (see Note 8). This is particularly important when the ELR monomer sequence is very repetitive (E and A blocks in our present case) but is not determinant when the ELR has a heterogeneous sequence in the middle of the monomer (CS5 peptide in REDV-ELR) used as the hybridization region. The melting temperature (T_m) of the complementary hybridization site should be higher than 60°C ($T_m (^{\circ}\text{C}) = 4 \cdot \text{GC} + 2 \cdot \text{TA}$).

3.1.2. Preparative PCR

Despite all the precautions taken during primer design, the monotonous character of these genes makes this amplification a delicate step. It is therefore advisable to first perform several analytic PCRs with a minimum volume to test the effect of variable of dNTPs, MgCl_2 , and primer concentrations or different annealing temperatures (T_a) (see Note 9). This procedure is described using construction of the REDV monomer gene (Fig. 1a) as an example:

1. Mix all the components of the PCR in a 0.2-mL, thin-walled tube on ice in the following order:
 - Nuclease-free water to final volume 50 μL .
 - 5 $\mu\text{L} \times 10$ *Pfu* buffer.
 - 2.5 μL REDV for primer (stock 10 μM) (optimal final concentration range 0.5–1 μM).
 - 2.5 μL REDV rev primer (stock 10 μM) (optimal final concentration range 0.5–1 μM).

- 2 μ L dNTPs Mix (stock 10 mM) (always after the buffer).
 - Mix by pipetting.
 - 2.5 U *Pfu* DNA polymerase (always last to be added).
 - Spin down the mix and hold on ice (see Note 10).
2. Put the samples in the thermocycler once its temperature is stabilized at 95°C (“Good Start procedure”) and run the following program:
 - First cycle: 5 min denaturalization at 95°C.
 - 20 cycles: 45 s denaturalization at 95°C.
 - 30 s annealing (see Note 11).
 - 45 s elongation at 72°C.
 - 15 min final elongation at 72°C.
 3. Separate a 1/5 aliquot of PCR samples on a high resolution 2–3% agarose gel (MetaPhor) in TAE buffer (agarose and buffer of choice for small DNA fragment recovery). After staining with 1 $\times$ SYBR green I solution visualize the bands by exposition to a UV lamp (Fig. 1b).

3.1.3. Cloning and Purification of ELR Monomeric Gene

In many cases, it is better to purify the PCR products using a preparative agarose gel instead of standard PCR clean-up kits before cloning due to the presence of nonspecific PCR fragments.

1. Load the rest of the PCR sample onto a preparative agarose gel. This is similar to the previous one with small specific characteristics. The comb has to be scaled to ensure separation of the whole sample. The gel should be run slowly (50 V for 3 h) to allow correct separation of the bands and, after staining, it should be visualized for a shorter time under a low power UV lamp to avoid DNA mutations.
2. Excise the slice of agarose containing the band from the gel with the help of a scalpel. A minimum quantity of agarose should be removed during band extraction.
3. Purify the monomer genes from the agarose with the “QIAquick Gel Extraction Kit” system, following the manufacturer’s instructions. Elute the DNA in 20 μ L of warm elution buffer.
4. Clone the purified band with the “Blunt-Ended PCR Cloning Kit” system and transform the competent *E. coli* cells. Plate cells on LB-agar culture dishes supplemented with antibiotic and incubate overnight at 37°C.
5. Select individual clones by colony screening PCR and agarose gel analysis (see Note 12). Prepare the PCR reaction mix in ice using external oligonucleotides (standard sequencing primers) to the polylinker of the cloning plasmid and the *Taq* DNA polymerase in a volume of 15 μ L. Touch a single colony with a sterile toothpick (or yellow tip) in a flow chamber, and dip it into PCR tube and shake. Perform the PCR, adapting the

annealing and elongation times and temperatures to the size of the expected genes and type of polymerase. Run the samples in a 1.5–2% analytical agarose gel and select the positive clones on the basis of the length of the resulting bands.

6. Grow the selected transformants overnight in 10 mL LB medium supplemented with antibiotic (250 rpm, 37°C). Extract their plasmids using the BioRad miniprep kit and elute the DNA in 50 µL of warm elution buffer. The length of the insert can be tested by restriction mapping using *Eae*I or co-digestion with *Eco*RI and *Hind*III endonucleases and an analytical agarose gel.
7. Examine the selected plasmids (pMOS::REDVmon) by automated DNA sequence analysis to confirm the correctness of their inserts sequence.

3.2. Synthesis of ELR Polymeric Genes: Concatemerization Method

The first strategy to obtain the polymeric genes is the concatemerization method, a random, directional concatenation of the monomeric gene. This method allows multimeric genes with a defined distribution and discrete lengths to be obtained in a single cloning cycle. The first step involves obtaining a large and concentrated amount of monomer fragments by type IIS endonuclease digestion. REDVmon contains two *Eae*I restriction sites flanking its ORF. These sites are eliminated from the excised fragment after *Eae*I digestion and the nonpalindromic protruding bases generated (GTA/CAT), which leads to unidirectional seamless concatenation (Fig. 1d). The second step is the concatemerization reaction itself, which is performed in a controlled ligation reaction where fragment purity and concentration are crucial factors. Finally, the concatemerization mixture is directly cloned in an expression vector. It is often necessary to tailor commercial expression vectors to clone the concatemerization product. In our case, we customized the pET25 vector by consecutive site-directed mutagenesis to remove internal *Sap*I sites and modify its multiple cloning site region to obtain a suitable vector (see Note 13).

1. Digest 90 µg of plasmid pMOS::REDVmon with 40 U of *Eae*I endonuclease (see Note 14) by overnight incubation at 37°C (see Note 15).
2. Purify the resulting gene fragment in a preparative 2–3% (Fig. 1c) agarose gel as described previously.
3. Perform a standard DNA precipitation to improve DNA fragment purity and concentration. Add 0.1 volumes of 3 M sodium acetate pH 5.2 (store at 4°C), 1 mL of 20 mg/mL glycogen (store at 4°C), and 2.5 volumes of cold 100% ethanol (store at –20°C). Leave overnight at –20°C for total recovery (see Note 16). Centrifuge the sample for 15 min at highest speed in a centrifuge at 4°C. Wash the DNA twice with cold 70% ethanol

(store at -20°C). Resuspend the pellet in the desired volume of type I water to achieve a concentrated solution (about $100\text{ ng}/\mu\text{L}$). Store at -20°C if not required immediately.

4. Prepare the concatenation reaction on ice as follows: $50\text{ ng}/\mu\text{L}$ of REDVmon fragment, 400 U of T4 DNA ligase in $40\text{ }\mu\text{L}$ of $1\times$ T4 DNA ligase reaction buffer. Incubate the mix overnight at 4°C .
5. Analyze a $1/10$ aliquot of ligation product in a 1.5% agarose gel (Fig. 1e) to verify the multimer pattern produced (see Note 17).
6. Digest $10\text{ }\mu\text{g}$ of mutated pET25 (pET25^{mut}) with 10 U *Sap*I endonuclease in $100\text{ }\mu\text{L}$ of $1\times$ reaction buffer overnight at 37°C .
7. Check vector cleavage by analyzing a $1/10$ aliquot of the sample in a 1% agarose gel.
8. Add 5 U of SAP to the digestion mixture and incubate for 1 h at 37°C . Inactivate SAP at 70°C for 15 min and store at 4°C .
9. Purify the dephosphorylated linear pET25^{mut} in a preparative 1% agarose gel as described previously. After purification, check the concentration and purity by agarose electrophoresis.
10. Add 150 ng of purified pET25^{mut} to 500 ng of concatenation mixture in a minimum volume ($10\text{ }\mu\text{L}$) and incubate overnight at 4°C (see Note 18).
11. Transform competent *E. coli* cells with $1\text{--}5\text{ }\mu\text{L}$ of ligation product. Plate cells on LB-agar culture dishes supplemented with antibiotic and incubate overnight at 37°C .
12. Analyze by colony-screening PCR, restriction analysis (Fig. 1g), and examine by automated DNA sequence analysis of individual clones, as described previously.

This concatemerization method has allowed us to synthesize a library of REDV-ELR clones with different repetitions of a unique REDV monomer (Fig. 1a) in a single procedure.

Although this technique offers a powerful approach to obtain multimerized genes, it nevertheless has some drawbacks. First of all, the impossibility of controlling the order or number of repetitions – if control of the monomer addition sequence is required then iterative–recursive methods must be used instead (see below). A second limitation concerns the presence of circular multimers of various lengths as by-products. In general, however, the amount of circular DNA in the concatenation mixture is not a major problem as, although it reduces the efficiency of the subsequent cloning step, it cannot be integrated.

3.3. Iterative–Recursive Method

The identity and sequence of the individual blocks within the polymer dictate the nature of the supramolecular assembly. To obtain reliable results it is therefore necessary to have absolute control

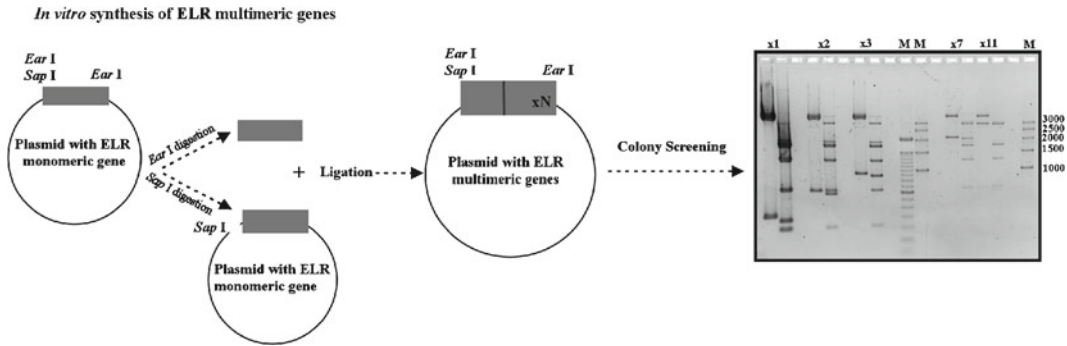


Fig. 2. Schematic representation of the iterative-recursive method for the synthesis of multimeric ELR genes.

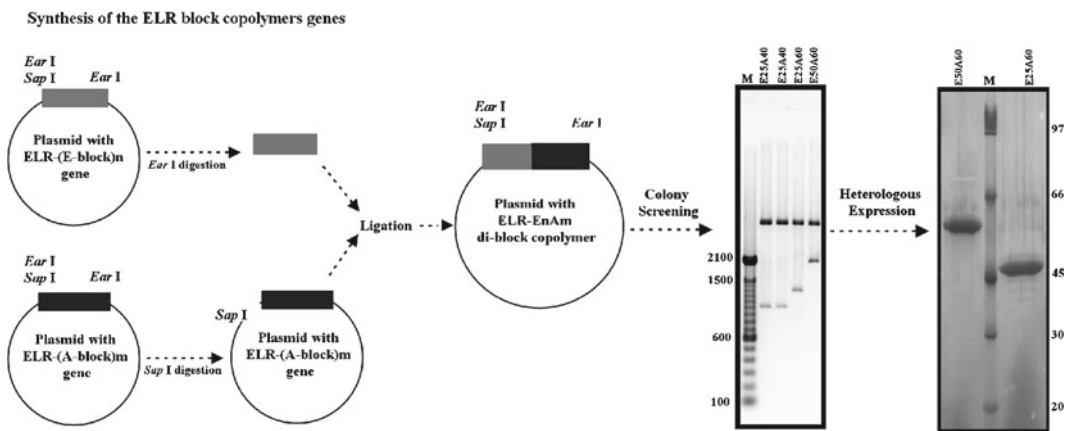


Fig. 3. Schematic representation of the iterative-recursive method for the synthesis of amphiphilic ELR block co-polymers.

over the polymer's sequence. The iterative-recursive method fits this need perfectly as it allows complete control of the size and distribution of the individual blocks that play central roles in formation of the supramolecular structures. This technique allows both polymeric genes (Fig. 2) and amphiphilic di- and tri-block copolymer genes to be synthesized (Fig. 3), although it has two important prerequisites. First of all, the ELR genes should be flanked by two *EarI* restriction sites, in order to split the genes by action of this endonuclease, one of which must also be a *SapI* restriction site (whose action will result in linearization of the receptor vector formed by the cloning plasmid and the ELR gene). Secondly, all the receptor vectors must lack endogenous *SapI* restriction sites (see Note 19).

3.3.1. Synthesis of ELR Polymeric Genes

Construction of ELR homopolymeric genes can be performed randomly using the concatemerization method, as described above, or in a controlled manner using the recursive method. Although this step-by-step procedure is very time-consuming method, it allows tight control of the resulting products, which is often

imperative when the construction of perfectly defined blocks is required. Below we describe dimerization of the ELR A-block as an example (Fig. 2).

1. Digest the pDrive:ELR-(A-block) (mutated pDrive plasmid lacking endogenous *SapI* sites and containing the A-block) with *EarI* and *SapI* endonucleases in two different tubes. Use 1 μ g of plasmidic DNA and 1 U of *SapI* endonuclease for vector linearization. For monomer gene production, take 5 μ g of plasmidic DNA and 5 U of *EarI* endonuclease and leave both restriction enzymes overnight at 37°C.
2. Add 5 U of SAP phosphatase to the *SapI*-containing tube and incubate for 1 h at 37°C, then transfer the sample to a 70°C water bath for 15 min and cool in ice.
3. Isolate the restriction products from a preparative agarose gel as described previously. Use the *SapI*-treated sample to purify the dephosphorylated linear plasmid including a copy of the A-Block monomeric gene, and isolate just the ELR gene from the *EarI*-treated sample (see Note 20). After purification, check the concentration and size of the DNA fragments with an analytical agarose gel.
4. Perform a DNA ligation by adding 50 ng of the isolated vector, 150 ng of pure monomer gene, 1 U of T4 DNA ligase, 1 μ L of T4 DNA Ligase Buffer, and water to a final volume of 10 μ L (see Note 21). Incubate overnight at 4°C.
5. Transform competent *E. coli* cells with 1–5 μ L of the ligation mixture. Plate cells on LB-agar culture dishes supplemented with antibiotic and leave overnight at 37°C.
6. Analyze individual clones by colony-screening PCR, as described previously.
7. Grow the selected transformants in 10 mL of LB medium supplemented with antibiotic.
8. Extract the plasmids using the Quantum Prep Plasmid Mini Kit and elute the DNA in 50 μ L of warm elution buffer. Prepare two reaction mixtures in ice, one with 0.2 U of *EarI* endonuclease, 1 μ L of buffer, and up to 10 μ L of water, and the other with 0.2 U of *EcoRI* endonuclease, 1 μ L of buffer, and up to 10 μ L of water. Add 10 μ L of reaction mixture and 5 μ L of eluted plasmid DNA to cold Eppendorf tubes and leave to digest for 3 h at 37°C. Run the samples on a 1.5% agarose gel. Figure 2 shows the analysis of the plasmids with the polymeric ELR-(A-block) genes. Restrictions with *EcoRI* produce two bands, an upper band corresponding to the pDrive plasmid and a lower one corresponding to the multimeric gene. Digestion with *EarI* leads to gene fragment liberation along with other bands from the pDrive vector.

Application of this recursive method therefore results in the synthesis of polymeric genes starting from a monomeric gene of a certain size. In this particular case, the monomer gene is the pentapeptide (VPGAG)₂₀ and all the resulting polymeric genes are repetitions of $20 \times n$ (where $n = 1, 2, 3, 7$, and 11).

3.3.2. Synthesis of Amphiphilic ELR Copolymer Genes

It has been reported that some of the physical properties of ELR copolymers are dependent on their architecture or block distribution and hence on the gene sequence (17). It is therefore necessary to determine which block in the di-block copolymer is going to be at the polypeptide N terminal before starting the experimental procedure. Although any possible distribution of the blocks can be obtained, in this example the hydrophilic block (ELR-E-block) is present at the N terminal and the hydrophobic block (ELR-A-block) at the C terminal of the polypeptide, i.e., E-A di-block or E₅₀A₆₀ considering the number of pentapeptides. The experimental protocol is similar to that described in Subheading 3.3.1.

1. Incubate the plasmid pDrive::ELR-(A-block)₃ with *SapI* endonuclease and the pDrive::ELR-(E-block)₁₀ with *EarI*. If the pDrive::ELR-(A-block)₃ is linear, it must be dephosphorylated to avoid self-ligation in the next step.
2. Isolate the digestion products – the (E-block)₁₀ fragment and the pDrive::ELR-(A-block)₃ plasmid – as described above and prepare a DNA ligation reaction with a 1:1 vector:insert molar ratio.
3. Transform competent *E. coli* cells and perform colony screening by PCR or restriction mapping with the appropriate restriction enzymes for the isolated plasmids. Figure 3 shows the restriction analysis with *EcoRI* endonuclease on four ELR copolymer genes. The sizes of the lower bands show the existence of genes encoding the polymers E₂₅A₄₀, E₂₅A₆₀, and E₅₀A₆₀ (the first two constructs were prepared in a different experiment).
4. Subclone the E₅₀A₆₀ ELR copolymer gene into the pET25^{mut} expression vector as described above.

This cloning strategy using a combination of *SapI* and *EarI* endonucleases allows the synthesis of several types of ELR di-block copolymer genes that can be used as receptor vectors or insert producers in new subcloning cycles to manufacture complex multiblock ELRs in just a few steps.

3.4. ELR Expression and Purification

ELR purification is based on a molecular transition of the polymer chains known as the inverse temperature transition (ITT), which is characteristic of this kind of genetically engineered protein polymer. In aqueous solution, and below a certain transition temperature (T_t), the free polymer chains remain disordered in the form of random coils that are fully hydrated, mainly by hydrophobic

hydration. Above T_i , however, the chain loses essentially all of the ordered water structures and hydrophobically folds and assembles to form a phase-separated state in which the polymer chains adopt a regular structure known as a β -spiral, which is stabilized by hydrophobic contacts. This is known as the ITT. The hydrophobic association grows to form particles several hundred nanometers in diameter before settling into a visible phase-separated state. This folding is completely reversible on lowering the sample temperature below T_i , the value of which depends on the molecular mass, the mean polarity of the polymer, and the presence of other ions and molecules (see Note 22). The ELR therefore precipitates upon raising the temperature above T_i , whereupon it can be separated from the remaining bacterial proteins by centrifugation and resolubilized by adding cold water and slow stirring. This protocol describes the expression and purification of the E-A diblocks, which is identical to REDV-ELR preparation.

1. Grow a single colony of *E. coli* BLR(DE3) harboring pET25^{mut}::ELR gene for 6 h at 37°C in an orbital shaker at 250 rpm in 5 mL of LB medium with antibiotic.
2. Reinoculate 2 mL of the resulting culture in 50 mL of fresh LB-amp medium and allow to grow overnight at 37°C at 250 rpm.
3. Inoculate 2 L of fresh LB-amp medium and allow to grow at 37°C in 2 L flasks (500 mL LB in each) to an optical density of 0.6–0.7 at 600 nm.
4. Induce the ELR expression with IPTG (0.5 mM) for 4 h at 37°C (see Note 23). Figure 1f (lanes 2–5) shows the SDS-PAGE analysis of the time-course induction of REDV-ELR (5).
5. Harvest the cells by centrifugation (6,500 $\times g$, 15 min at 4°C) and discard the supernatant. Wash the cells twice by resuspension in TBS and further centrifugation.
6. Resuspend the bacteria in 1 $\times$ SB buffer and disrupt using a 2-s sonication pulse every 5 s at 100 W. Keep the samples on ice to avoid protein denaturation and limit protease action (see Note 24). Centrifuge the suspension at 15,000 $\times g$ for 60 min at 4°C and keep the supernatant. The absence of ELR in the cellular debris (insoluble fraction) should be checked by SDS-PAGE (see Note 25).
7. Adjust the pH of the soluble fraction to 3.5 by the addition of dilute hydrochloric acid; this step must be done on ice and with vigorous shaking. Remove the denatured material (acid proteins and DNA) by cold centrifugation at 15,000 $\times g$ for 20 min. Keep the supernatant and ensure that the pH remains acidic (see Note 26).
8. Heat the sample at 60°C for 2 h followed by a warm centrifugation for 20 min at 15,000 $\times g$.

9. Resuspend the pellet in 2 mL of cold type I water. Neutral pH values help the polymer re-suspension process, therefore addition of dilute NaOH is often necessary. Shake for 2 h on ice to compensate the hysteresis effect of the A-block.
10. Repeat the procedure (steps 7–9) twice more while checking the pH values and the resuspension time on ice as these two factors dramatically influence the final yield of polymer.
11. Check the ELR purity by SDS-PAGE electrophoresis after staining with CuCl_2 (see Note 27). Isolated REDV-ELR is shown in Fig. 1f (lane 1), whereas purified polymers $\text{E}_{25}\text{A}_{60}$ and $\text{E}_{50}\text{A}_{60}$ are shown in Fig. 3 (see Note 28).

3.5. Biomedical and Nanotechnological Applications of ELRs

3.5.1. Construction of Bioactive Scaffolds with the REDV-ELR

ELRs have been used to produce different kinds of two- (18) and three-dimensional (19–21) tissue-engineering scaffolds. Thus, film- or layer-type supports facilitate the visualization and analysis of cell–material interactions, whereas the microstructure of ELR-based hydrogels, which present very high surface and internal porosity, simulates the structure of the ECM.

We have produced highly transparent 2D REDV-ELR films and 3D hydrogels that maintain the polymer's mechanical properties (20) (Fig. 4). Both REDV-ELR scaffolds are formed by covalent

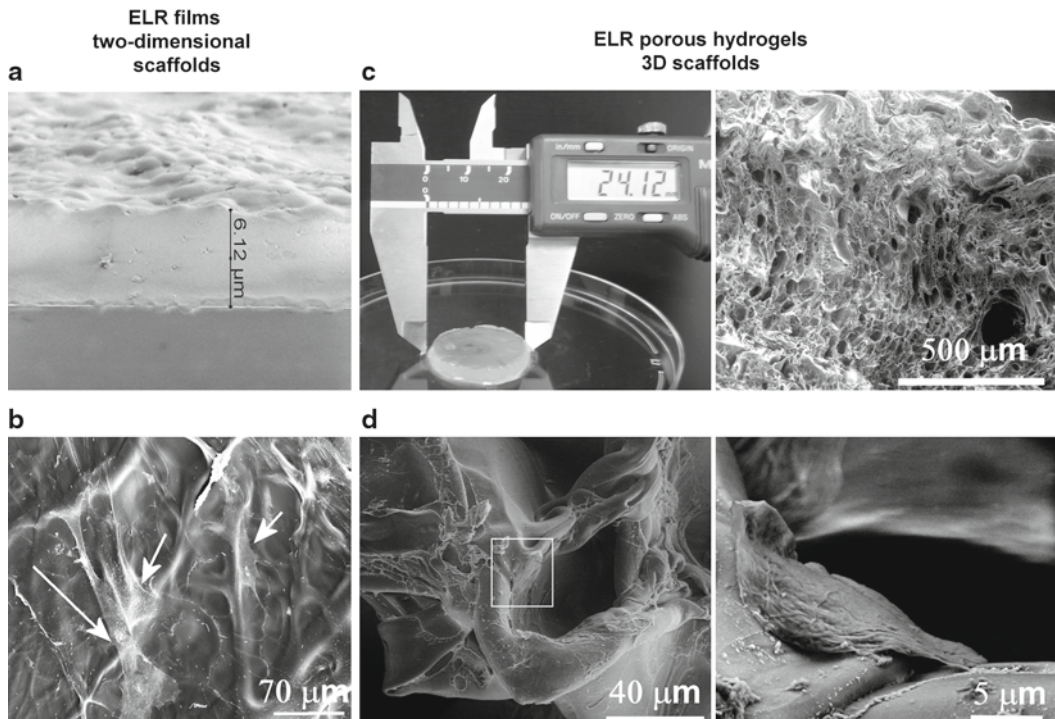


Fig. 4. 2D and 3D REDV-ELR scaffolds. Microscopy images of endothelial cells (HUVECs) seeded on chemically cross-linked ELR supports (20, 21). (a) Scanning electron micrographs of cross-linked films. (b) Cells seeded on REDV-ELR films. (c) Macroscopic pictures of swollen hydrogels in water and SEM micrograph of its cross-section. (d) Cross-sectional SEM micrographs and its magnified view shows the presence of cells within the internal pores.

chemical cross-linking between a homobifunctional HDI reagent and the ϵ -amine groups of the polymer's L-lysine moieties.

The film-manufacture protocol involves solvent casting deposition and subsequent cross-linking.

1. Deposit a 50-mg/mL aqueous REDV-ELR solution on a glass support and dry at 60°C for 8 h.
2. Dip the support in a 10% (v/v) HDI/acetone solution overnight at room temperature.
3. Wash the film thoroughly with type I water.
4. Sterilize by UV exposure overnight and store in 70% ethanol. Wash with sterile type I water and PBS before cell culture assay.

3D hydrogels were cast into molds by mixing a solution of REDV-ELR and cross-linker reagent in an organic solvent.

1. Mix 25 mg/mL HDI/DMF solution with REDV-ELR (80 mg/mL) dissolved in DMSO:DMF (80:20) (final polymer/crosslinker molar ratio of 1:3) and cast in cold Teflon molds for 3 h at room temperature.
2. Wash and sterilize as above.

On REDV-ELR films and hydrogels have cultured endothelial cells (HUVECs) with a well-spread morphology (Fig. 4) (20, 21).

3.5.2. Self-Assembly of Diblock ELR Copolymers

Amphiphilic ELR copolymers exhibit a two-step thermal response whereby the copolymer solution is transparent at low temperatures but the absorbance increases and remains well above baseline levels as the temperature increases. Upon further heating, the solution turbidity increases sharply before plateauing at a constant value. It is believed that the first small increase in absorbance is caused by the ITT of the ELR block with the lowest T_t (the A-block in this particular case). Upon heating above a determined temperature, the high- T_t ELR block (E-block) undergoes its ITT, which induces the formation of large aggregates, as indicated by the high OD values. The turbidity assays allow the formation of supramolecular structures to be checked indirectly as well as the behavior of the block copolymers when heated and cooled.

1. Prepare a 25- μ M di-block ELR solution in cold water, adjust the pH to 7, and leave on ice for 30 min to ensure polymer solubilization (see Note 29).
2. Perform the OD measurements at 350 nm, starting at 5°C and increasing the temperature by 0.5°C every 5 min (see Note 30).

Figure 5 shows the turbidity profile for the E₅₀A₆₀ di-block copolymer (21). An increase in turbidity is a first sign of self-assembled supramolecular structure formation, which should be verified subsequently using specific techniques.

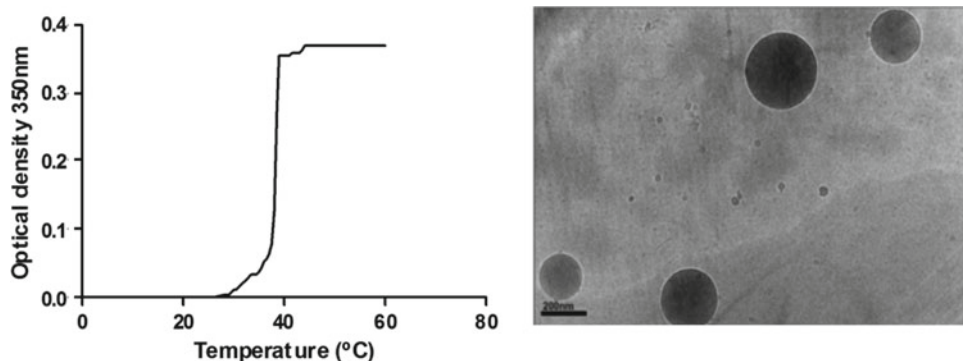


Fig. 5. Turbidity profile and cryo-TEM image of the $E_{50}A_{60}$ di-block ELR (21).

Cryo-TEM is a direct method that gives conclusive information regarding the type of self-assembled nanostructures obtained from multiblock copolymers at temperatures higher than the polymer's ITT.

1. Prepare a 1-mg/mL di-block copolymer solution in cold type I water (see Note 31). Stabilize the samples at 4°C and 50°C for 30 min prior to measurements (see Note 32).
2. Perform the cryo-TEM experiments. Figure 5 shows an image of the nanoparticles formed by the $E_{50}A_{60}$ ELR copolymer at 50°C; no recognizable structure was observed at 4°C.

An analysis of these images clearly shows the inherent ability of this recombinant amphiphilic ELR to form supramolecular structures (21). The diameter of this di-block copolymer (250 ± 15 nm) suggests the formation of vesicular structures when solutions of this polymer are heated above its temperature transition.

4. Notes

1. dNTP mix aliquot are stored at -20°C. Defrost on ice before use.
2. Leave the agarose to hydrate for at least 20 min before melting. Melt at low heating power and avoid boiling.
3. Defrost cells on ice before use.
4. Add ampicillin just before inoculation.
5. *E. coli* strain recommended for use with tandem repeat peptides.
6. In the last few years several companies (GenScript, Genentech, Sloning BioTechnology among others) have begun to offer the synthesis of any gene with no limits to its complexity, length, or scale. We have used some of these, although with contrasting results due to their repetitive structure.

7. Several type IIS restriction enzymes (*Pfl*MI, *Bgl*II *Bpi*II, *Eco*3II, *Bbs* I, *Bsm* BI, etc.), all of which can be used for the seamless cloning of ELRs with excellent results, are available. This particular enzyme pair was chosen on the basis of their recognition and restriction sequences and their widespread availability.
8. Although codon usage does not appear to be an essential parameter for high production levels, higher yields were obtained in some cases by taking into consideration the specific codon bias. Codon usage tables for *E. coli* can be found at:
 - <http://www.kazusa.or.jp/codon/> (Accessed June 2010)
 - <http://www.geneinfinity.org> (Accessed June 2010)
 - pET System Manual – 11th Edition – Novagen
9. To achieve the expected amplification we suggest modifying the concentration of some PCR components in parallel (primers/template in the range 0.1–1 mM in increments of 0.2 mM; dNTPs in the range 100–250 μ M) taking into account that higher specificity and lower yields are obtained at lower concentrations. $MgCl_2$ concentration also influences both yield and fidelity in inverse proportion. The standard concentration for this reagent is 2 mM, but the range 1–3 mM in 0.5 mM increments can be tested.
10. The preparation of two different negative-control PCR samples lacking one of each primer is recommended.
11. Hot Start or Touchdown techniques can often be used to improve the specificity of this amplification of extremely repetitive genes. There are several Hot Start amplification systems on the market. In the Touchdown technique, the program starts with an initial annealing temperature (T_a) of 72°C, 10–12°C above the expected melting temperature (T_m) of the hybridization zone of oligonucleotides. T_a is then progressively reduced every two PCR amplification cycles until the predetermined annealing temperature is reached, then an additional ten cycles are carried out at this temperature. The elongation time is chosen to be close to the minimum period required for sequence amplification.
12. PCR screening of the recombinant colony is a powerful method for positive clone identification. However, it can be inefficient for genes that encode ELRs as they are composed of very repetitive sequences with a high cytosine/guanine content, which means that the DNA melting temperature of the codifying sequence is higher than the annealing temperature for conventional primers. Consequently, part of the codifying sequence itself may act as a primer during PCR amplification in a nonspecific priming phenomena that significantly impedes PCR sensitivity and results in unspecific multiple products that smear on a gel. In our experience, the presence of a regular ladder of amplified products is a sign of the existence of repetitive genes.

13. pET25 vector mutations are generated by PCR using the “Quick Change Site-directed Mutagenesis Kit” and pairs of oligonucleotide primers designed with mismatching nucleotides at the middle of the primers. The *g* and *h* primers should be used to eliminate vector endogenous *SapI* recognition sites without modification of the resulting ORFs and SitelinkerpETPhos primers (*i* and *j*) should be used to modify the multiple cloning site. We have obtained multiple mutations with a unique site-directed mutagenesis cycle: addition of seamless cloning recognition sites; inclusion of GTA codons by unidirectional ligation; insertion of the translation stop codon; deletion of the pelB leader sequence and substitution of some amino acid residues at the N terminal.
14. Many commercial cloning plasmids contain multiple type II endonuclease recognition sites and therefore result in extensive fragmentation. It is therefore often advisable to perform a simultaneous digestion with a suitable endonuclease (*DpnI*, *MseI*, etc.) which prevents the co-purification of unspecific fragments with a similar length without affecting the ELR gene.
15. To determine the starting amount of plasmid to digest, the amount of insert with respect to the plasmid size (in our case around 10%) and the efficiency of the separation and purification methods (in our experience both around 80–90%) should be calculated.
16. The addition of an inert carrier such as glycogen increases DNA recovery in dilute or short DNA samples.
17. The expected result is a ladder with defined bands corresponding to the size of the multimers (Fig. 1e). The band-size analysis shows that both linear and circular DNA multimers can coexist. When circular DNA limits the cloning efficiency, the linear type can be promoted by increasing the concentration and purity of the DNA starting fragments.
18. The insert:vector molar ratio is higher than for standard subcloning protocols as its molar ratio cannot be calculated exactly here because the inserts are a distribution of multimers. A successful cloning mix with a molar ratio of 60:1 was prepared by considering the inserts as monomers.
19. Site-directed mutagenesis of the vectors containing the individual blocks has to be performed in order to use the iterative–recursive method to generate polymeric genes. In our particular case, mutation is dependent on the presence of endogenous *SapI* restriction sites on the cloning vector (pDrive) and was performed as described in Note 9.
20. It is advisable to purify the linearized plasmid from the gel because the presence of even a minute amount of undigested plasmid can complicate the following steps. An alternative

method for purifying extremely long linearized plasmids is the “Ultrafree-DA” (Millipore) filtration system. The filtered DNA should be precipitated as described above.

21. A vector–insert molar ratio of 1:5 should normally be used on account of their sizes. This ratio can be changed depending on the number of multimeric genes. A 1:1 molar ratio can be used when a simple duplication of the gene is required, although the resulting transformants will be fewer in number.
22. The presence of two residues (L-alanine and L-glutamic acid) with different properties in the $E_{50}A_{60}$ ELR affects the purification protocol. The hydrophobic A-block is pH-independent and shows hysteresis during cooling solubilization, whereas the hydrophilic E-block is pH-dependent since the γ -carboxylic group of the L-glutamic acid residue changes its state with the pH, thus affecting the T_t of the recombinamer.
23. Alternatively, ELR production can be performed with Circle Grow Media (Q Biogene) or the Overnight Expression System (Novagen).
24. In our experience, ELRs are usually very resistant to bacterial protease degradation, although the bioactive domains in some tissue engineering ELRs have been shown to be protease sensitive. In this case, we use protease inhibitors or reduce the induction temperature. Two common and inexpensive inhibitors are PMSF and EDTA.
25. The buffers used during purification have a pH close to neutral, where the γ -carboxylic group ($pK_a \approx 4.5$) of the L-glutamic acid residue is in its carboxylate form. This significantly increases the polymer’s polarity and thus T_t , thereby affecting the final yield of the purification process.
26. The γ -carboxylic group of the L-glutamic acid residue is protonated under these conditions therefore the ITT of the E-block occurs at lower temperatures. The T_t of the E-block in $E_{50}A_{60}$ ranges from 33°C at pH 3.5 to >70°C at pH 7 (15).
27. The extremely hydrophobic amino acids of the elastomeric domains present in the ELR backbone are responsible for the failure of most of the conventional staining methods for protein detection in SDS-PAGE. The common choice for ELRs is copper chloride-based negative staining. Briefly, wash the gel with distilled water for 10 s and incubate for 5 min in a fresh 0.3 M $CuCl_2$ solution. The salt excess can be removed with distilled water. Another advantage of this fast technique is the fact that all the salt can be rapidly removed by incubation in an EDTA solution and the gel can be restained.
28. The observed rate of migration of ELRs in SDS-PAGE does not agree with their theoretical molecular weight: it is usually higher than the theoretical size, with a maximum increase in

band position of 20%. This unusually high electrophoretic mobility is typical for this type of hydrophobic polymer and is even more evident when the polypeptide sequence includes an L-alanine residue.

29. It is important to allow the samples to stabilize with agitation before starting to measure to ensure the absence of nonsolubilized polymer aggregates.
30. This 5 min period is key at higher temperatures as the supramolecular structures start to form at these temperatures and the elapsed time is essential for their stabilization.
31. Filter the copolymer solutions to remove nonresuspended polymer.
32. The maximum value is dependent on the copolymer's ITT, which should be at least 5°C higher than the polymer's transition temperature to ensure the stabilization of the supramolecular structures.

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