Elastin-like polymers as nanovaccines: protein engineering of self-assembled, epitope-exposing nanoparticles

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Abstract

In this chapter we describe two unconventional strategies for the formulation of new nanovaccines. Both strategies are based on obtaining chimeric genes that code for proteins in which the major antigens of the pathogens are fused to an elastin-like recombinamer (ELR) as carrier. ELRs are a family of synthetic protein biopolymers obtaining using DNA recombinant techniques. The ELRs employed in the present chapter are block copolymers that are able to assemble, under controlled conditions, into nanoparticles similar to virus-like particles and to provoke an immune response. We describe the biosynthesis of ELRs genetically fused to an antigenic sequence from *Mycobacterium tuberculosis* and a simple procedure for obtaining stable nanoparticles displaying the antigen in the first strategy. The second approach describes the production of a DNA vaccine library consisting of plasmids codifying for major antigens from Rift Valley fever virus fused to different ELR-based block copolymer architectures.

The procedures described can be adapted for the production of other chimeric DNA-protein vaccines based on protein polymer carriers.

1. Introduction

The current global pandemic has highlighted the importance of developing effective and safe vaccines using alternative and versatile procedures and has accelerated the scientific and social acceptance of historically unconventional vaccines. The series of vaccines developed using different genetic-engineering approaches clearly shows the possibilities of developing innovative, safe and effective vaccines that comply with strict and rigorous authorization procedures and safety regulations at all times [1]. Apart from the antigen chosen, the design of the carrier or adjuvant molecule is also of great

importance for the success of the prospective vaccine [2]. Some of the most interesting alternative carriers for vaccines include the recombinant production of protein backbones that spontaneously form the desired macrostructures and which can be genetically complemented with antigenic fragments [3, 4].

During the design of these recombinant protein devices, multicomponent chimeric genes with a careful balance between the gene encoding the structural part or carrier responsible for automatically forming virus-like particles, fused with the antigenic peptide that should be displayed on the device's surface, are constructed. The chimeric genes may be expressed in a heterologous expression system to obtain the multifunctional polypeptides for administration as protein nanoparticles [3], or cloned into a plasmid vector suitable for RNA-DNA vaccination in mammalian hosts [5].

Elastin-like polymers (ELPs) are bioinspired, synthetic biopolymers derived from selected motifs from one of the extracellular matrix proteins, namely natural elastin. Almost all of them comprise repeat motifs from the elastomeric domains of elastin, such as the pentapeptide Val–Pro–Gly–Xaa–Gly, as a monomeric component of their sequence (the guest residue (Xaa) can be any naturally occurring L-amino acid except proline) [6]. The monotonous molecular structure of ELPs confers a set of interesting properties, such as stimuli-responsiveness, biocompatibility and self-assembling behavior,[7] but also technical difficulties that hinder their synthesis [8, 9]. The recombinant production of elastin-like polymers has allowed the generation of large numbers of specific ELPs whose complexity at a molecular level and accuracy and reproducibility of production means that this technique has supplanted other synthetic methods. These biopolymers obtained using genetic-engineering techniques are commonly referred to as elastin-like recombinamers (ELRs) [10].

The potential of ELRs in the biomedical field as advanced biomaterials or therapeutic agents, including fusion partners with antigens for the development of nanovaccines that induce a similar or better immune response than that found for more traditional vaccines, has been widely explored. ELRs have been synthesized to combat many different diseases. The different ELR-based vaccines include the porcine circovirus type 2 antigen of the capsid protein-based virus-like particle (VLP) vaccine for porcine circovirus-associated disease[11, 12], which generates higher protection than commercial vaccines, or the M2e influenza antigen fusion ELR [13], both of which are produced in *Escherichia coli*. Furthermore, plants have also been developed as

expression hosts, for example the fusion of Ag85B/ESAT-6 antigens to ELRs for the development of a subunit vaccine against tuberculosis disease [14], or the fusion of ELRs to the beak and feather disease virus (BFDV) capsid protein for a subunit vaccine against Psittacine beak and feather disease [15], both of which have been successfully produced in tobacco plants. ELRs have also been studied as cytotoxic T lymphocyte (CTL) vaccine carriers, with promising results [16, 17].

The purpose of this chapter is to describe two different vaccination strategies employing ELRs as vaccine carriers and the corresponding protocols.

2. Materials

2.1 Reagents

-1 Kb Plus DNA Ladder (Invitrogen)

-β-Mercaptoethanol (Sigma-Aldrich)

-Ampicillin (Apollo Scientific)

-Bromophenol Blue (Sigma-Aldrich)

-dNTPs mix (Stratagene)

-DNA primers (Metabion)

-*Dpn*I (ThermoFisher Scientific)

-*Ear*I (*Eam*l 104I) (ThermoFisher Scientific)

-*Eco*RI (ThermoFisher Scientific)

-EndoFree Plasmid Maxi Kit (Qiagen)

-Ethylenediamine Tetraacetic Acid (EDTA) (Sigma-Aldrich)

-FastAP phosphatase (Fermentas)

-FBS (Invitrogen)

-Glutamine (Invitrogen)

-Glycerol (Sigma-Aldrich)

-*Hind*III (ThermoFisher Scientific)

-Penicillin/Streptomycin (Invitrogen)

-*Pfu* Turbo DNA Polymerase (Agilent)

-PureLink Quick Gel Extraction Kit (Invitrogen)

-Quantum Prep Plasmid Miniprep Kit (Biorad)

-QuickChange Site-Directed Mutagenesis Kit (Stratagene)

-*Ear*I (ThermoFisher Scientific)

-*SapI*(*Lgu*I) (ThermoFisher Scientific)

-Seakem agarose (Cambrex)

-Shrimp alkaline phosphatase (SAP) (Fermentas)

-Simply Safe nucleic acid stain (Eurx)

-SOC I (Sigma-Aldrich)

-T4 DNA ligase (Fermentas)

-XL1-Blue (Stratagene): endA1 supE44 hsdR17 thi1 recA1 gyrA96 relA1 lac [F' proAB lacIq ZΔM15 Tn (Tetr)]s

-3,3',5,5'-Tetramethylbenzidine (TMB, 1-StepTMUltra TMB-ELISA)

2.1.2 Materials for analysis of Gn glycoprotein in vitro expression

-293T: Human embryonic kidney cells (ATCC)

-Black-/clear-bottomed 96-well plates ((ThermoFisher Scientific)

-Diamidino-2-phenylindole dihydrochloride (DAPI) (Lonza)

-Dulbecco's modified Eagle's medium (DMEM) (Invitrogen)

-FBS (Invitrogen)

-Glutamine (Invitrogen)

-Goat anti-mouse Alexa Fluor 488 (ThermoFisher Scientific)

-Lipofectamine LTX Plus Reagent (Invitrogen)

-Milicell® EZ Slides (Merck Millipore)

-Paraformaldehyde (PFA) (Sigma-Aldrich)

-Penicillin/Streptomycin (Invitrogen)

-Phosphate Buffered Saline (PBS) (Invitrogen)

-RVFV-immunized mouse serum (Kindly given by Dr. Brun)

-Triton X-100 (Sigma-Aldrich)

-Turbofect Transfection Reagent (Fermentas).

2.1.3 Materials for analysis of in vivo biodistribution in mice

-293T Human embryonic kidney cells (ATCC)

-BALB/c mice aged 12-16 weeks (Janvier Labs).

-Anhydrous *N*,*N*-dimethylformamide (DMF) (Sigma-Aldrich)

-Ethanol (molecular biology grade, Thermo Scientific)

-Isoflurane (Esteve)

-Psoralen-PEG_3-Biotin (Thermo Scientific)

-Qdot® 800 streptavidin conjugate (Invitrogen)

-PBS (Invitrogen)

-Potassium acetate (Sigma-Aldrich)

2.2 Buffers and media

-DNA loading buffer 5x: 30% (v/v) glycerol (Sigma-Aldrich), 0.1% (w/v) SDS (Sigma-Aldrich), 0.05% (w/v) bromophenol blue (Sigma-Aldrich), 50 mM Tris (Sigma-Aldrich) pH 8, 0.05 mM EDTA (Sigma-Aldrich)

-LB-Agar: 25 g/L LB (Pronadisa) + 1.5% (p/v) Agar (Fluka)

-Luria Broth (LB): 25 g/L (Pronadisa).LB and LB-Agar are sterilized on an autoclave for 20 min at 120°C and 1 atmosphere.

- Modified Terrific Broth media 56 g/L mTB (Pronadisa). Sterilized in an autoclave at 120°C and 1 atmosphere for 20 min.

-TAE (pH 8): 40 mM Tris-acetate (Sigma-Aldrich) pH 8, 1mM EDTA.

-TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4)

3. Methods

3.1 ELR-vaccine device synthesis

3.1.1 Molecular design guidelines

For the development of ELR nanovaccines in which the antigen is exposed on the outer part of the nanoparticle as a fusion protein with the ELR backbone, an ELR block copolymer based on an amphiphilic structure comprising two diblocks was chosen. The hydrophilic block is a glutamic acid (E)-based polar block, while the hydrophobic block has the amino acid isoleucine (I) in the guest position. The hydrophobic block coacervates in water under physiologically relevant conditions (pH 7.4, 37°C), thereby maximizing hydrophobic interactions that drive self-assembly, whereas under the same conditions, the incorporation of charged hydrophilic residues, such as glutamic acid, prevents block aggregation. When building the ELR construct, certain aspects, such as

the location of the blocks when the nanoparticle is formed, have to be taken into account. Thus, the antigenic sequences must join to the terminus of the hydrophilic block of the combined hydrophobic-hydrophilic blocks so that they are exposed at the surface of the nanoparticle after self-assembly (**Fig. 1**).

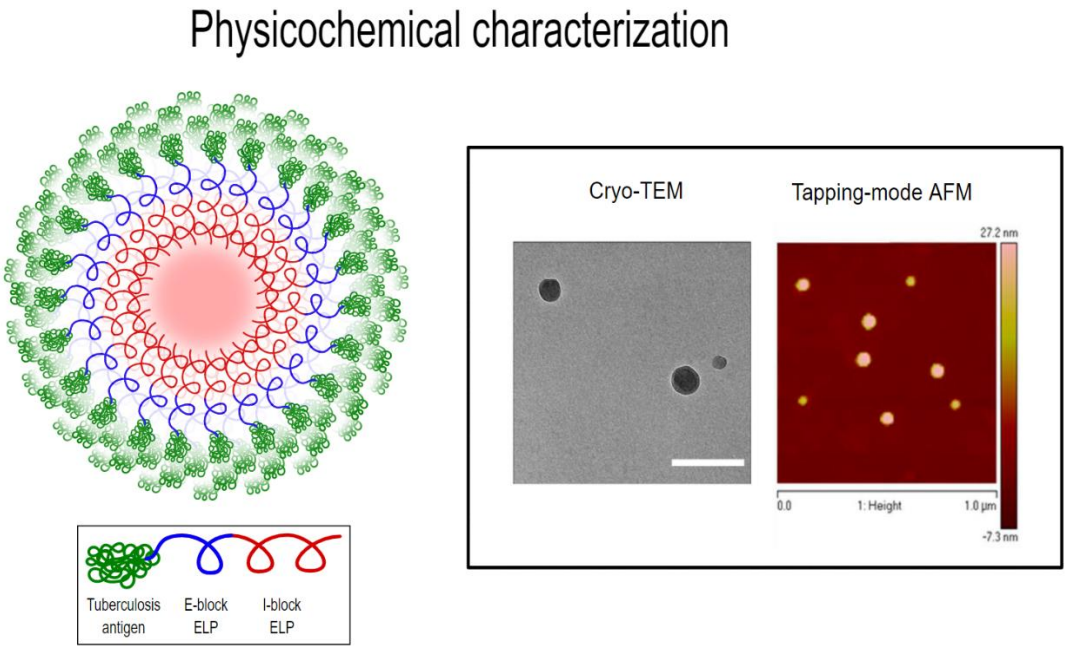


Figure 1. *Schematic representation of the chimeric protein nanoparticle comprising tuberculosis antigen, E-block and I-block. Physicochemical characterization of these self-assembled nanoparticles was carried out using cryo-TEM and tapping-mode AFM. The scale bar for the sample analyzed by cryo-TEM is 100 nm.*

3.1.2 Optimization of E. coli expression

Some considerations for optimization of *E. coli* expression include:

1. Minimizing the GC content at the 5'-end to avoid the formation of secondary structures in the mRNA, which leads to interrupted translation and low levels of expression.
2. Adding a transcriptional terminator, or an additional one if one is already present.
3. Avoiding codons that have been associated with translation problems, such as AGG, AGA CGG CGA for arginine; GGA for glycine, AUA for isoleucine, CUA

for leucine and CCC for proline. Another approach is the use of *E. coli* strains that encode some of them.

4. Examining the second codon. Differences in expression of up to 15-fold have been found depending on the codon after the first methionine. The most used is AAA lysine[10].
5. Addition of a fusion partner
6. Use of protease-deficient host strains. For example, BL21 is deficient in two proteases encoded by the *lon* (cytoplasmic) and *omp*T (periplasmic) genes.

3.1.3 Chimeric gene construction

The hydrophobic block, which has the sequence $(VGIPG)_{60}$, provides a mechanical behavior similar to natural elastin. In addition, the γ-carboxyl groups in the $[(VPGVG)_2$-(VPGEG)-$(VPGVG)_2]_{10}$ hydrophobic block, undergo strong polarity changes between the protonated and deprotonated states. As a consequence of changes in pH around its effective p*K*a, the Tt values of this block are directly affected by the pH. The E-block does not show any phase transition at pH values above its p*K*a (4.1), but can affect the Tt of block-copolymers associated with it. The result of this combination is a block copolymer that self assembles into multimeric nanoparticles or hydrogels, depending on the concentration, at room temperature before reaching physiological conditions (see Note 1).

Sequential introduction of repetitive ELR polypeptides codifying gene segments to form fusion genes with a fully controlled composition and chain length is carried out using the “recursive directional ligation” (RDL) strategy, which allows the monomer genes to polymerize in a seamless and unidirectional manner. This seamless cloning requires the use of type IIS restriction enzymes, which recognize asymmetric sequences and cleave DNA outside their recognition site. This feature makes them suitable for polymer biosynthesis by guaranteeing unidirectional ligation and avoiding the addition of extraneous amino acids that could alter the properties of the final polymer product. To enable application of the RDL to obtain ELR-based polymeric genes, the sequence has to be flanked by *Ear* I recognition sites at both ends and by one *Sap* I restriction site coinciding with the 5’EarI site of the gene, which can be used for linearization of the cloning vector and as a source for gene insertion. In brief, the same plasmid can be

opened with *Sap* I or the gene insert extracted by digesting with *Ear*I as the insert and plasmid have been designed to have compatible ends (see Note 2).

Construction starts with a pDrive cloning vector bearing the $E_{50}I_{60}$ block, which has been previously constructed by RDL (see Note 3).

1. Plasmid linearization is achieved by digestion of 3 µg of pDrive plasmid containing the $E_{50}I_{60}$ block with 3 U of *Sap I* in a final reaction volume of 50 µL under the suggested reaction conditions.
2. To ensure that the plasmid has been correctly and exhaustively digested, and to avoid future problems during cloning, we recommend to perform an analytic electrophoresis on 1% agarose in TAE buffer (see Note 4).
3. To avoid self-ligation of the linearized plasmid, the adequate amount of Shrimp Alkaline Phosphatase (SAP) phosphatase should be added, along with the corresponding buffer, incubating at 37°C for 1 h. To calculate the amount of SAP needed, remember that 1 pmol of DNA ends is equivalent to about 1 µg of a 3 kb plasmid (see Note 5).
4. Inactivate the SAP enzyme by incubating at 70°C for 15 min and then cool to 4°C.
5. Add the corresponding volume of DNA loading buffer to the sample (see Note 6).
6. Separate the linearized plasmid by preparative DNA electrophoresis on 1% agarose in TAE buffer (see Note 7) at a fixed voltage of 60 V for approximately 90 min (see Note 8).
7. Isolate the linearized plasmid band visualized under the UV light transilluminator from the preparative agarose gel by cutting it and physically removing with a scalpel. Avoid unnecessary exposure of the DNA to the potential mutagenic action of UV light.
8. Extract the DNA of interest from agarose by melting in chaotropic buffer in a 50°C water bath and apply to the spin-column of a commercial Gel extraction system.
9. Measure the DNA concentration using a UV-VIS spectrophotometer (see Note 9).
10. Select the antigen sequence and synthesize its sequence flanked by *EarI* sites, then clone in a suitable plasmid.
11. To obtain a sufficient quantity of the cloning plasmid bearing the antigen sequence, it should be transformed into competent *Escherichia coli* for XL1-Blue cloning. The competent cells and the BD Falcon polypropylene tubes must be

placed on ice before thawing so that the cells are aliquoted directly into the pre-chilled tubes. The highest efficiency for the transformation of XL1-Blue super-competent cells is achieved after a heat pulse of 45 seconds (see Note 10). Heat pulses of less than 30 seconds or more than 45 seconds result in lower efficiencies. After the recovery incubation, plate in LB agar with the corresponding antibiotic.

12. Incubate overnight at 37°C for 16-18 hours to allow the transformants to form isolate colonies. Higher incubation times can result in the development of satellites colonies.
13. To screen the clones bearing the correct plasmid, select the desired number of transformant colonies and grow each of them overnight in 5 mL LB medium supplemented with the appropriate antibiotic (250 rpm, 37°C).
14. Extract the plasmids from the bacterial cultures using the alkaline lysis method.
15. Measure the plasmid concentration using a UV-VIS spectrophotometer.
16. To isolate the sequence of the antigen gene from the plasmid, digest 5 μg of plasmid containing the hapten (immunogenic sequence) with 5 U EarI at 37°C for 6 hours in a final reaction volume of 50 μL (see Note 11). The amount of plasmid to be digested also depends on the size of the DNA encoding the hapten: shorter fragments are difficult to visualize.
17. Control an aliquot of the digestion mixture in an analytic gel of appropriate concentration to check for complete and correct digestion before performing preparative electrophoresis.
18. The apten gene is isolated by preparative electrophoresis at 3V/cm for approximately 90 min or until the band of interest is completely separated. The mobility of the loading buffer dyes helps to estimate the position of the DNA bands.
19. Isolate the band for the gene of interest from the preparative agarose gel by cutting it with a scalpel in an ultra-violet (UV) transilluminator and purify the DNA fragment of interest as described above.
20. Measure the insert concentration using a micro volume UV-VIS Spectrophotometer.
21. Perform a ligation reaction with pDriveE50I60 *SapI* linearized plasmid and *Ear*I digested insert (1:5 molar ratio) with at least 70 ng of plasmid and the

corresponding quantity of insert, 1 U of T4 DNA ligase, 2 μL of T4 ligase buffer and a final volume of 20 μL (see Note 12). Incubate at 22 °C for 1 h or at 4°C overnight.

22. Transform 5 μL of the ligation reaction mixture (up to 50 ng of the experimental DNA) into *Escherichia coli* strain XL1-Blue and plate in LB agar with the appropriate antibiotic (see Note 13), incubating the transformants at 37 °C overnight or at room temperature for 48 h.
23. Once the colonies have grown, select the desired number of transformant colonies and grow each of them overnight in 5 mL LB medium supplemented with antibiotic (250 rpm, 37°C).
24. Extract their plasmids using the alkaline lysis method.
25. Correct insertion of the gene fragment can be tested by restriction mapping using *EarI* or *EcoRI* endonucleases and an analytical agarose gel. Restrictions with *EcoRI* produce two bands: a band of approximately 4000 bp corresponding to the pDrive plasmid and another corresponding to the gene. Digestion with EarI leads to gene fragment liberation along with other bands from the pDrive vector.
26. After performing restriction mapping, verify the selected plasmids by DNA sequence analysis to confirm the correctness of their sequence.
27. When the desired complete sequence is achieved, subclone the final gene into a modified pET-25(+) expression vector. Briefly, extract the gene from pDrive using *EarI*, as in previous steps, and ligate with *SapI* linearized and dephosphorylated pET-25(+), as described previously for pDrive, at a 1:1 molar ratio (see Note 14).

3.1.4 ELR expression and purification: Isolation of a highly productive colony

Before starting production of the ELR of interest, it is necessary to determine the expression level of several clones of the expression bacterial strain containing the same final plasmid DNA to select the best producer. Colony selection is an important procedure to achieve a high-yield production of proteins. To that end, it is recommended to perform two small-scale production screenings consecutively to identify the most productive colony.

1. To obtain expression bacterial clones of the final plasmid DNA, transform the final construct into *E. Coli* competent BLR(DE3) cells and culture on an LB-agar plate (see Note 15). Follow the appropriate transformation protocol (see Note 16).
2. Analyze the expression rate of the protein of interest with respect to the total protein fraction by performing a simple induction test. To that end, several colonies are analyzed, each one being used to inoculate 5 mL of auto-inducible modified TB medium (mTB) supplemented with the antibiotic of interest (see Note 17) until the appropriate final concentration of 1% (see Note 18).
3. Culture each inoculum at 37°C with shaking (250 rpm) for 16 hours (overnight). An appropriate amount of biomass is generated under these conditions and expression of the protein of interest is induced.
4. Analyze a 1 mL sample from the cultures grown for each colony by polyacrylamide gel electrophoresis, as described below:
5. Separate the bacteria from the culture medium by centrifuging the samples at 12000 g and a temperature of 4 °C for 1 min; discard the supernatant.
6. Wash the bacterial pellet by adding 1 mL of ultrapure water type I or 1X PBS (see Note 19) to the pellet and resuspend it with a vortex (see Note 20).
7. Repeat steps 5 and 6 to obtain a pale pellet.
8. To observe a suitable concentrated band pattern, resuspend the pellet in 200 µL of ultrapure water type I with a vortex.
9. Remove a 20 µL aliquot and mix with 5 µL of 5X protein loading buffer to denature native proteins into unfolded rod-like structures with a uniform negative charge density per unit mass. This charge affects the electrophoretic mobility of each polypeptide formed (see Note 21).
10. Boil the mixture for 5 min and after 5 min spin-down, load 10 µL of the sample onto an SDS polyacrylamide gel (SDS-PAGE) with an appropriate %T (see Note 22). Heating the samples for a few minutes in the presence of a reducing agent such as 2-mercaptoethanol helps to denaturalize proteins completely by reduction of the disulfide bonds.
11. Perform the electrophoresis at 1 mA per cm and select the colony that produces the most intense band for the protein of interest with respect to the bacterial proteins; this is the most productive colony.

12. Increase the quality of the producer colony by performing a second screening between best-producer-colony "children". To that end, plate the most productive colony and grow it on an LB-agar.
13. Repeat steps 2 to 11 and select the most productive colony again (see Note 23).

3.1.5 ELR production

Once a highly productive colony for the polymer of interest has been obtained, the next step is to produce the ELR.

14. To ensure an appropriate quantity of bacterial biomass, the selected *E. coli* BLR(DE3) colony is cultured in sequential steps. Firstly, in a 50 mL tube, a pre-inoculum is obtained in 5 mL of LB supplemented with the required antibiotic and glucose, until a final concentration of 1% (v/v) is obtained (See note 24). Culture overnight at 37°C with shaking (250 rpm).
15. Subsequently, 100 µL of the resulting pre-inoculum culture is inoculated in 30 mL of fresh antibiotic/glucose-supplemented LB broth in a 150 mL Erlenmeyer flask (see Note 25). Culture at 37°C and 250 rpm for 6 h until the exponential phase is reached (OD600 = 0.6–0.7) (See Note 26).
16. A high yield of recombinant protein expression is achieved with mTB medium supplemented with the appropriate antibiotic. Prepare the desired volume of medium for total production culture and inoculate with 1 mL of the last inoculum (step 15) per 500 mL of broth (see Note 27).
17. Culture at 37°C and 250 rpm overnight until the stationary phase is reached. At that point induction has finished and the stationary phase of the bacterial growth curve has been reached (see Note 28).
18. Stop metabolism and growth by cooling the cells to 4°C with the help of an ice bath.
19. Verify that the protein of interest has been expressed correctly by analyzing the total protein fraction composition during the induction time-course on a polyacrylamide electrophoresis gel.
20. Harvest the cells by centrifugation at 4°C and 5000 g for 15 min. Discard the supernatant and wash the cells from the pellet by resuspension in 100 mL of TBS wash buffer per liter of culture, shaking energetically. It is important to keep the temperature low with ice (see Note 29). The washing step should be repeated until

the supernatant is colorless and the pellet is clear, which shows the absence of residual medium.

21. Prepare the bacteria for lysis. Firstly, resuspend the bacterial pellet in 25 mL of TE sonication buffer per liter of culture (see Note 30).
22. To avoid bacterial protease activity during the purification steps, add 2 mL of 10 µg/mL phenylmethylsulfonyl fluoride (PMSF) protease inhibitor to the cell suspension per liter of added TE buffer and maintain at 4°C.
23. Bacteria are lysed by performing 7-14 sonication cycles, each cycle comprising pulses of 10 seconds each every 15 seconds at 100 W for 30 min. (see Note 31)
24. Separate solid bacterial debris from the sonicated suspension by centrifugation at 4°C and 15000 g for 90 min. Store the two phases, considering that the supernatant should contain the soluble polymer while the pellet is the insoluble phase (see Note 32).
25. Check which phase contains the protein of interest by SDS-PAGE: collect 1 mL of sample, centrifuge and test the protein content of each phase in an SDS-PAGE assay. Commence the purification process with the phase selected.

3.1.6 ELR purification

ELR purification is based on the smart nature of these biomaterials, more specifically the inverse temperature transition phenomenon (ITT) typical of ELRs, which can be triggered by heat, changes in pH or addition of salt. This procedure involves a simple method called Inverse Transition Cycling (ITC) which allows recombinant proteins and peptides to be purified (Fig. 2). Although a standard ITC protocol has been established for chimeric proteins, each target protein requires some degree of prior optimization.

1. Facilitate the separation of soluble contaminants by acidification of the soluble fraction to pH 4 with dilute hydrochloric acid. Denatured acid proteins and DNA are removed by cold centrifugation at 4°C and 15,000 g for 20 min. Discard the pellet and keep the supernatant, which should contain the protein of interest.
2. Recover the polymer by triggering its phase transition by increasing the solution temperature to 40°C (above the ELR inverse transition temperature) for 2 h in a warm water bath. At this temperature the ELR forms micrometer-sized aggregates, which can be separated from the soluble fraction of the cell lysate by

centrifugation at 15,000 g for 20 min. Decant and discard the supernatant (*see* Note 33).

3. Soluble contaminants and insoluble proteins can co-localize in the solid fraction with the ELR. As such, resuspend the pellet in 2 mL of cold ultrapure deionized water per litre of culture, and leave to stir at 4°C for 12 hours.
4. Insoluble contaminants trapped in the ELR aggregates are removed as precipitates by centrifugation at 15,000 g for 30 min.
5. The ELR-containing soluble fraction is decanted and retained, thereby completing the ITC cycle.
6. Repeat the purification process (steps 25–29) several more times to increase the purity of the ELR protein (*see* Note 34).
7. Freeze sample, eliminate water and dry by lyophilization.

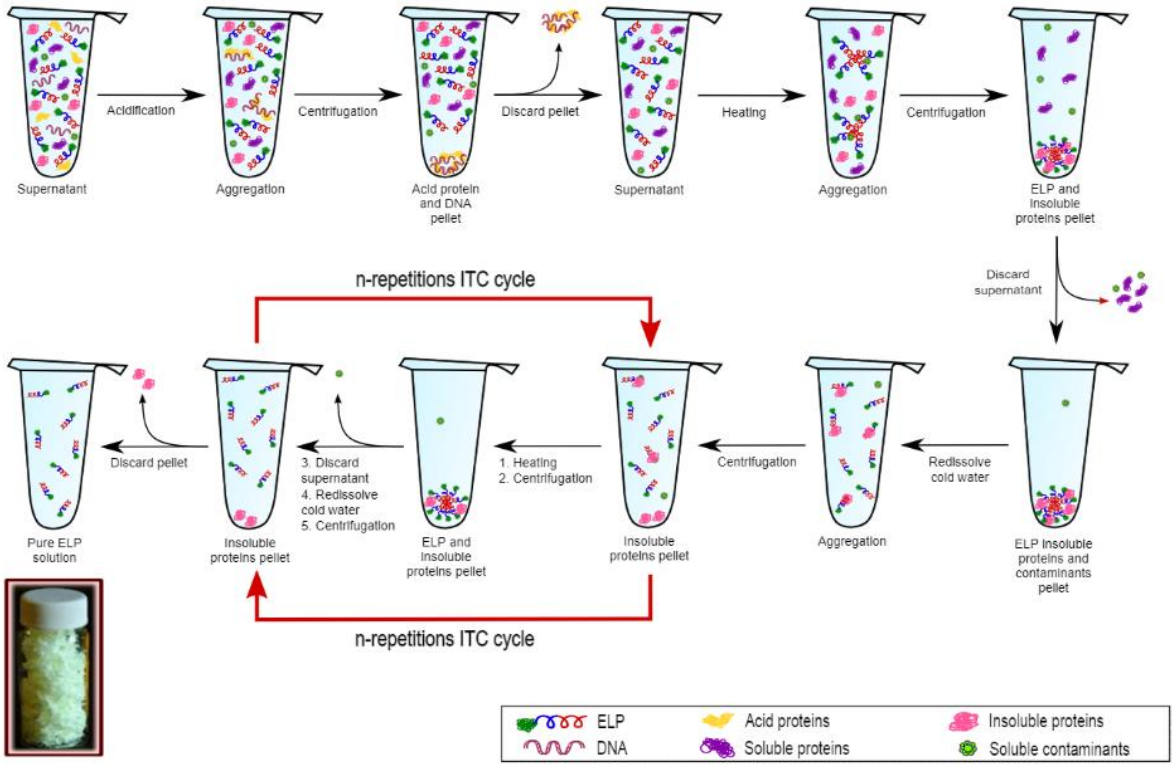


Figure 2: Scheme showing the purification of recombinant proteins using the Inverse Transition Cycling (ITC) method. In order to obtain completely pure recombinant proteins, *n* ITCs are carried out in such a way that the chimeric polymer is obtained free from contaminants.

3.1.7 Endotoxin removal from ELRs

Endotoxins are complex lipopolysaccharide from the outer membrane of Gram-negative bacteria, such as *E. coli*, typically found as contaminants in recombinant proteins. Their structures comprise three subdomains covalently linked to each other, and they exhibit strong activity. As a result, endotoxin-contaminated nanomaterials have shown toxic effects in *in vivo* or *in vitro* transfection studies, thereby confounding the results [18]. Consequently, before starting *in vivo* or *in vitro* immunosafety assays, endotoxin levels must be evaluated and these substances removed, if present, by way of a secondary treatment with sterile sodium hydroxide (NaOH) and sodium chloride (NaCl).

8. Precipitate the ELRs from the last cold supernatant at 25°C by adding sterile 10 N NaOH to a final concentration of 0.4 N and mixing vigorously.
9. Incubate the mixture on ice for 15 min, then add 5 M NaCl to a final concentration of 2 M.
10. Collect the protein by centrifugation at 25°C and 8500 g for 20 min. Discard the supernatant and resuspend the pellet in sterile PBS at approximately 50 mg per 20 mL.
11. Repeat steps 33–35 three times. After the third treatment, adjust the solution pH to 6-8.
12. Dialyze the last cold supernatant with cold ultrapure water, using 0.2 mm filters, until the salts have been completely removed. The number of dialysis cycles required can be estimated by considering that two equilibria against a 1000-fold volume excess of buffer will decrease the salt concentration 10^6-fold; three dialyses under these conditions 10^9-fold, *etc*.
13. Freeze the sample, eliminate the water and dry by lyophilization.
14. Weigh the ELR obtained, calculate the yield achieved and store at -20°C.
15. Determine endotoxin levels in the purified ELR by resuspending it at serial dilutions, from 1 mg/mL, in sterile molecular biology grade water.
16. Use a commercial assay to assay the protein endotoxin content. Levels of endotoxin should be typically ≤0.1 EU/mg of protein polymer (1 EU = 100 pg

of endotoxin), although the purity requirement depends on the route of administration, residence time and application.

Table 1. Endotoxin limit defined in the EUROPEAN PHARMACOPOEIA – EDQM 5.1.10. Guidelines for using the test for bacterial endotoxins. https://www.edqm.eu/sites/default/files/medias/fichiers/COVID-19/updated_covid-19_vaccines_package_oct_2020.pdf

Route of administration	***K***
Intravenous	5.0 IU of endotoxin per kilogram of body mass
Intravenous for radiopharmaceuticals	2.5 IU of endotoxin per kilogram of body mass
Intrathecal	0.2 IU of endotoxin per kilogram of body mass
Parenteral formulations administered per square metre of body surface	100 IU of endotoxin/m^2

17. Verify your ELR by physicochemical characterization. NMR and FT-IR spectroscopy can be used to verify the structure of the protein. Furthermore, amino acid analysis can be performed by HPLC, and the molecular weight can be obtained by MALDI-TOF. Turbidimetry and DSC allow the transition temperature of the protein to be calculated, whereas the nanoparticle size and zeta-potential can be studied by DLS. The loading of the different components can be evaluated by thermogravimetric analysis (TGA) or UV-vis absorbance, depend on their nature. The morphological characteristics of nanoparticles can also be determined using several microscopy techniques, such as TEM, SEM or AFM.
18. Introduce any chemical post-translational modifications required (*see* Note 35).

3.2 ELR-based DNA vaccines

Nucleic acid vaccines, namely DNA- or RNA-based vaccines comprising plasmids codifying for major antigens under the control of eukaryotic promoter sequences, have recently emerged as an encouraging alternative due to their

ability to induce both humoral and cellular immune responses, as well as high stability and easy design and manufacturing compared to conventional vaccines [19, 20]. Large libraries of tailor-made antigens can be designed and built using genetic-engineering techniques to generate enhanced immune responses in the host [21]. Rift Valley fever virus (RVFV) is a negative strand RNA virus (genus Phlebovirus, family Phenuiviridae, order Bunyavirales) that causes a severe disease in ruminants characterized by high rates of morbidity and mortality. This virus is transmitted in different ways, such as the bite of a mosquito, inhalation of aerosols and direct contact with bodily fluids from infected animals [22]. Although endemic in sub-Saharan Africa, Egypt and Saudi Arabia, the disease also has the potential to spread much further. Indeed, multiple cases have been reported recently in different region worldwide, such as the Arabian Peninsula, Madagascar and some Indian Ocean islands. Thus, the disease caused by RVFV may have unpredictable consequences for public and animal health in disease-free continents [23].

Although vaccination is the only effective strategy for the prevention of RVF, currently available vaccines present low immunogenicity and adverse side-effects. New vaccines are therefore needed to develop better and safer tools that are able to trigger a strong immune response.

In this section we describe the protocols followed by Gonzalez-Valdivieso et al. to establish a gene library of DNA nanovaccines specifically designed against the RVFV glycoprotein Gn, including genes encoding for ELR blocks [4]. Six chimeric fusion genes are described in that work, all of them including the gene sequence codifying for the Gn glycoprotein from RVFV and ELR blocks of a different nature.

3.2.1 Plasmid mutagenesis and cloning

Starting from a pCMV plasmid containing the gene codifying for the glycoprotein Gn under the control of cytomegalovirus (CMV) promoter, which is a potent promoter for attaining high gene expression (kindly provided by Dr. Alejandro Brun of Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Centro de Investigación en Sanidad Animal (CISA), Madrid), PCR site-targeted mutagenesis is performed to prepare the plasmid and

clone the genes codifying for different ELR blocks next to the gene encoding the viral Gn glycoprotein (see Note 36).

First, the endogenous *SapI* recognition site (5'-GCTCTTC-3') is removed by substitution of a cytosine for an adenine at position 3306 in the plasmid sequence (Tables 2-3, Fig. 3 A).

Starting from a pCMV plasmid containing the gene codifying for Gn glycoprotein:

1. Prepare the PCR reactions in ice:

-5 µL of 10× reaction buffer
-X µL (50 ng) of dsDNA template (See Notes 37)
-X µL (125 ng) of oligonucleotide forward primer
-X µL (125 ng) of oligonucleotide reverse primer
-1 µL of dNTP mix
-1 µL of *Pfu* Turbo DNA polymerase (2.5 U/µL)
-ultrapure deionized water type I to a final volume of 50 µL.

The preparation of a negative-control PCR sample lacking DNA template (replaced by ultrapure deionized water type I) is highly recommended.

2. Put the reaction tubes in the thermocycler and select the parameters described in Table 3:

3. After the PCR reaction, place the reaction tubes on ice for 2 minutes to cool the reaction to ≤37°C.

Table 2. Sequences of mutagenesis primers for the c.1295C>A mutation in the DNA sequence of pCMVNSmGn for removal of a *Sap*I restriction site, and the original and mutated sequences. Mutated nucleotides are depicted in red. The recognition site for *Sap*I is depicted in bold.

Mutation	**Primer**	**Sequence (5'-3')**	**Tm (°C)**
Point mutation	MutSappCMVFor	GCGTATTGGGCGCT**A**TTCCGCTTCCTCGAC	78.5
	MutSappCMVRev	GTCGAGGAAGCGGAA**T**AGCGCCCAATACGC	78.5
Mutation	**Sequence**	**DNA sequence (5'-3')**	
Point	Original	GCGTATTGGGC**GCTCTTC**CGCTTCCTCGCTC	

mutation	Mutated	GCGTATTGGGCGCTATTCCGCTTCCTCGCTC	

Table 3. PCR settings for the c.1295C>A mutation. The recommended duration of the elongation step at 68°C is 1 minute per kb of plasmid length.

Segment	Cycles	Temperature (°C)	Time (s)
1	1	95	30
2	12	95	30
		55	60
		68	360

The amplification mixture is digested with *Dpn*I restriction enzyme to eliminate the DNA template and used to transform XL1-Blue supercompetent cells.

1. Add 1 µL (10 U) of the *Dpn*I restriction enzyme directly to each amplification reaction tube.

2. Gently mix by pipetting up and down. Spin down the reaction mixture in a microcentrifuge for 1 minute and immediately incubate at 37°C for 1 hour to digest the parental (non-mutated) DNA.

3. Thaw the XL1-Blue supercompetent cells on ice. For each reaction, aliquot 50 µL of the supercompetent cells into a pre-chilled 14-mL round-bottomed polypropylene tube.

3. Add 1 µL of the *Dpn*I-digested DNA to the aliquots of the supercompetent cells.

5. Incubate the reaction mixture on ice for 30 minutes.

6. Heat the reaction mixture at 42°C in a water bath for 45 seconds and then place on ice for 2 minutes.

7. Add 450 µL of SOC medium (preheated to 42°C) and incubate the transformation reaction mixture at 37°C and 250 rpm for 1 hour.

8. Plate 50 µL of each transformation reaction on LB-Agar plates containing ampicillin for the plasmid vector.

9. Incubate the transformation plates at 37°C overnight in the dark.

10. Select isolated colonies.

11. Pick a single colony from a freshly streaked selective plate and inoculate a culture of 5 mL LB medium containing ampicillin. Incubate overnight in an incubator at 37°C and 250 rpm.

12. Extract the DNA using the BioRad Miniprep Kit according to the manufacturer's instructions and elute the DNA in 50 µL of elution buffer.

13. After DNA extraction, measure the absorbance at 260 nm to determine the DNA concentration and quality with a nanodrop (See Note 38).

14. Correct mutagenesis of the plasmids is analyzed by restriction mapping with *Ear*I. Restriction digestions of non-mutated plasmid pCMV-NSmGn with *Ear*I produce six bands of 280, 488, 992, 1033, 1100 and 1804 bp. Due to the mutation c.1295C>A, an *Ear*I/*Sap*I recognition site is removed from position 3306 and the fragments of 1100 and 1804 bp are fused into one fragment of 2904 bp. Fragments of 280, 488, 992 and 1033 bp remain unaltered.

15. Among the colonies rendering the expected bands, verify the positive colonies by DNA sequencing, as described above. Table 4 lists the sequencing primers that can be used to verify transformants' DNA.

Table 4. Sequences of sequencing primers and their melting temperature.

Primer	Sequence (5'-3')	Tm (°C)
CtGn For	TTATAGGGTGCTTAAGTGC	42
XhoI For	ATCCGGTACTCGAGGAAC	46
pCMV Rev	CCTGAACCTGAAACATAAAATG	46
Mid1Gn For	CGTGATGAAGACACACTGTC	45
Mid2Gn For	GCCTTTATGTGTAGGGTATG	44

Once successful removal of the *Sap*I recognition site is obtained, a new *Sap*I recognition site (5'-GAAGAGC-3') is introduced after the Gn glycoprotein codifying gene by a second site-directed mutagenesis cycle (Tables 5-6 and Figures 3 A). ELR-encoding genes can therefore be introduced next to the gene codifying for the RVFV glycoprotein Gn to form the final fusion gene constructs.

Starting from the pCMVNSMGn c.1295C>A mutated plasmid as described above:

1. Prepare the PCR reactions in ice:

-5 µL of 10× reaction buffer
-X µL (50 ng) of dsDNA template
-X µL (125 ng) of oligonucleotide forward primer
-X µL (125 ng) of oligonucleotide reverse primer
-1 µL of dNTPs mix
-1 µL of *Pfu* Turbo DNA polymerase (2.5 U/µL)
-ultrapure deionized water type I to a final volume of 50 µL

2. Put the reaction tubes in the thermocycler and select the parameters shown in Table 6:

3. After the PCR reaction, place the reaction tubes on ice for 2 minutes to cool the reaction to ≤37°C.

Table 5. Sequences of mutagenesis primers for c.2028_2029 insGTATGAAGAGCTAGGC mutation and the original and mutated sequences. The second mutation involves the insertion c.2028_2029insGTATGAAGAGCTAGGC into the Gn sequence to insert a *Sap*I restriction site and STOP codon. Mutated nucleotides are depicted in red. The recognition site for *Sap*I is depicted in bold.

Mutation	**Primer**	**Sequence (5'-3')**	**Tm (°C)**
Multiple nucleotides insertion	MutCterGnFor	GCCCCTATTCCTCGTCATGTATGAAGAGCTAGGCGGCCG	80.2
	MutCterGnRev	CGGCCGCCTAGCTCTTCATACATGACGAGGAATAGGGGC	80.2
Mutation	**Sequence**	**DNA sequence (5'-3')**	
Multiple nucleotides insertion	Original	TATTCCTCGTCATTAGGCGGCCGCGG	

	Mutated	TATTCCTCGTCATGTAT**GAAGAGC**TAGGCGGCCGCGG	

Table 6. PCR settings for c.2028_2029insGTATGAAGAGCTAGGC mutation. The recommended duration of the elongation step at 68°C is 1 minute per kb of plasmid length.

Segment	Cycles	Temperature (°C)	Time (s)
1	1	95	30
2	18	95	30
		55	60
		68	360

After PCR site-directed mutagenesis, the parental template pDNA is digested with *Dpn*I and XL1-Blue supercompetent cells are transformed. The plasmid DNA of these cells is purified and analyzed by restriction digestion following the protocols described above for the first mutagenesis cycle.

The second site-targeted mutagenesis (c.2028_2029insGTATGAAGAGCTAGGC) involves insertion of the 16 nucleotides “GTATGAAGAGCTAGGC” at position 2826 in the plasmid sequence. This mutation creates a new *Ear*I/*Sap*I recognition site at position 2826. The theoretical fragments for the final plasmid after digestion with *Ear*I are 280, 488, 620, 992, 1033 and 2300 bp. As such, the fragment of 2904 bp is split into two fragments of 620 and 2300 bp, respectively. After this mutation, the pCMVNSmGn plasmid is therefore ready for introduction of the ELR-encoding genes from a gene library to develop different constructs, thereby resulting in DNA nanovaccines with enhanced expression of Gn glycoprotein from RVFV in eukaryotic systems.

Finally, verify the positive colonies giving the expected bands by DNA sequencing to corroborate the presence of the new *Ear*I/*Sap*I site.

3.2.2 Synthesis of the DNA-vaccine library

Once the pCMV plasmid with the gene sequence encoding for the RVFV Gn glycoprotein has been successfully mutated to allow the introduction of ELR-encoding genes, sequential introduction of the repetitive polypeptide-coding gene segments to form fusion genes, with full control of composition and chain length, is carried out using the RDL (recursive directional ligation) technique (Figure 3B). The genes encoding for the A80, I80, E75, V84, V168 and SILK blocks are available from previous studies from the group and were constructed using the same procedure of DNA concatenation involving sequential rounds of RDL with the monomeric DNA sequences (Figure 3C).

1. The mutated plasmids are linearized and dephosphorylated following steps 1-9 in section 3.1.3 “Chimeric gene construction”.
2. During the cloning of DNA-vaccines, different ELRs genes are isolated as described in steps 14-21 in section 3.1.3 “Chimeric gene construction”. In this cloning, due to the size of the inserts, T4 ligation is performed in a 1:2 vector-to-insert ratio to ensure ligation of one insert molecule per vector.
3. Plate the transformed cells on LB-agar plates supplemented with ampicillin and incubate overnight at 37°C.
4. Select individual clones and grow the selected transformants in 5 mL of LB medium supplemented with ampicillin.
5. Extract the DNA using the Endo Free Plasmid Maxi kit according to the manufacturer’s instructions and determine the DNA concentration and quality (See Note 39).

6. Restriction mapping of fusion genes. The correct cloning of the final gene constructs is analyzed by restriction mapping with *Hind* III and subsequent DNA sequencing.
7. Analytical restriction mapping is performed by incubating the following reaction mixture at 37°C for 2 h:

   -X µL (500 ng) of DNA
   -0.2 µL (1 U) of *Hind*III
   -0.5 µL of buffer
   -ultrapure deionized water type I to a final volume of 5 µL
8. Load the samples on a 1% agarose gel and run the DNA electrophoresis at 10 V/cm for 90 min. Fragments resulting from incubation with the *Hind*III restriction enzyme are depicted in Table 10.
9. Among the colonies rendering the expected bands, verify the positive colonies by DNA sequencing.
10. Determine the endotoxin levels of the final gene constructs using the Endosafe-PTS™ test
11. **Table 7.** Size of fragments resulting from incubation of fusion genes with *Hind*III restriction enzyme.

Gene construct	Size (bp)
wtGn	13; 1075; 4525
Gn-A80	13; 2275; 4525
Gn-I80	13; 2275; 4525
Gn-V84	13; 2335; 4525
Gn-E75	13; 2200; 4525
Gn-V168	13; 3595; 4525
Gn-V168-SILK	13; 3787; 4525

3.2.3 Protocol for analysing Gn glycoprotein expression in vitro

Expression of the chimeric polypeptides in the eukaryotic cells is evaluated by transfecting 293T human embryonic kidney cells. 293T cells are cultures in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin.

At the time of transfection, cells should have 70-80% confluence. Use black clear-bottomed 96-well plates to prevent signals from spreading between wells during the fluorescence measurements. The number of cells, quantities of DNA and reagent volumes are optimized for 293T cell cultures in 96-well-plates and 200 µL as final volume. Prepare fresh transfection reagent immediately before the transfection assay.

Lipofectamine® transfection:

1. Seed 293T cells in 96-well plates (2 x 10^4 per well) and incubate at 37°C and 5% CO_2 for 24 hours.
2. Dilute 2 µL of Lipofectamine® LTX Reagent in 25 µL non-supplemented medium.
3. Dilute 2.5 µg of DNA in 125 µL of serum-free DMEM.
4. Add 2.5 µL of PLUS™ reagent in diluted DNA.
5. Add 25 µL of diluted DNA (with PLUS™ Reagent) in 25 µL of diluted Lipofectamine® LTX Reagent.
6. Incubate at room temperature for 5 minutes.
7. Add 10 µL of the transfection mixture to each well.
8. Incubate at 37°C for 24 hours in a CO_2 incubator.
9. Replace cell medium with supplemented medium.
10. Analyze transgene expression after 24 hours by immunocytochemistry and confocal microscopy.

Turbofect™ transfection:

1. Seed 293T cells in 96-well plates (2 x 10^4 per well) and incubate at 37°C and 5% CO_2 for 24 hours.

2. Dilute 0.1 μg of DNA in 20 μL of serum-free DMEM.

3. Vortex the Turbofect™ reagent and add 0.4 μL to the diluted DNA.

4. Mix immediately by pipetting up and down.

5. Incubate at room temperature for 15 minutes.

6. Add 20 μL of the transfection reagent/DNA mixture to each well dropwise.

7. Gently rock the plate to achieve an even distribution of the complexes immediately after adding the transfection reagent.

8. Incubate at 37°C for 24 hours in a CO_2 incubator.

9. Replace the cell medium with supplemented medium.

10. Analyze transgene expression after 24 hours by immunocytochemistry and confocal microscopy.

Immunocytochemistry analysis

1. Seed 293T cells in 96-well plates (2 x 10^4 per well) and incubate at 37°C and 5% CO_2 for 24 hours.

2. Transfect cells as described above.

3. After 24 hours, wash with PBS 1X (200 μL per well), fix with PFA 4% (200 μL per well) for at least 30 minutes and wash three times with PBS 1X (200 μL per well).

4. Permeabilize cells with Triton X-100 0.1% in PBS (200 μL per well) for 10 minutes and wash three times with PBS 1X (200 μL per well).

5. Block with FBS 2% for 1 hour at room temperature.

6. Incubate cells with RVFV-immunized mouse serum for 1 hour at 37°C (See Note 40).

7. Wash three times with PBS 1X (200 µL per well).

8. Incubate with Alexa Fluor 488-labeled secondary antibody 5 µg/mL for 1 hour at 37°C (200 µL per well).

9. Measure the fluorescence intensity from the bottom using a microplate reader.

Confocal microscopy

1. Seed 293T cells in Milicell® EZ Slides (2 x 10^4 per well) and incubate at 37°C and 5% CO_2 for 24 hours.

2. Transfect the cells as described above.

3. After 24 hours, wash with PBS 1X (500 µL per well), fix with PFA 4% (500 µL per well) for 30 minutes and wash three times with PBS 1X (500 µL per well).

4. Permeabilize cells with Triton X-100 0.1% in PBS (500 µL per well) for 10 minutes and wash three times with PBS 1X (500 µL per well).

5. Block with FBS 2% at room temperature for 1 hour.

6. Incubate cells with RVFV-immunized mouse serum at 37°C for 1 hour.

7. Wash three times with PBS 1X (500 µL per well) and incubate with Alexa Fluor 488-labeled secondary antibody at 37°C for 1 hour (500 µL per well).

8. Wash cells with PBS 1X (500 µL per well).

9. Stain cell nuclei with DAPI for 5 minutes and wash with PBS 1X (500 µL per well).

Avoid prolonging the DAPI incubation time to prevent an excessively high signal from cell nuclei and merged images due to incorrect contrast between channels.

3.2.4 Protocol for analysis of biodistribution in mice in vivo

Fluorescent labeling of DNA constructs.

1. Adjust DNA to 100 µg/mL in TE buffer.

2. Dissolve Psoralen-PEG_3-Biotin in DMF at 20 mM.

3. Add the biotin solution to the DNA and mix well by pipetting up and down.

4. Irradiate the reaction tube from above with a 15W UV lamp at 365 nm for 30 minutes on ice.

5. Precipitate the sample with 0.2 M potassium acetate and two volumes of 70% ethanol to remove unreacted biotin.

6. Centrifuge at 5000 rpm for 15 min.

7. Discard the supernatant, wash the pellet with 400 μL 70% ethanol and allow it to dry.

8. Dissolve the biotinylated sample in sterile PBS.

9. Dissolve the Qdot® 800 streptavidin conjugate in Secondary Incubation Buffer at 40 nM.

10. Add the Qdot® 800 streptavidin conjugate to biotinylated DNA and incubate at room temperature for 15 minutes. Use a 2:1 plasmid DNA:Qdot® 800 molar ratio to allow all the quantum dots to be complexed with the plasmid DNA.

Intravenous administration of fluorescently labelled DNA in mice.

1. Inject the fluorescently labelled DNA into BALB/c mice (2.5 mg/Kg mouse) intravenously via the tail vein. Use the diluted Qdot® 800 streptavidin conjugate immediately in the current experiment.

2. Anaesthetize the mice with isoflurane in oxygen (4%) in an induction chamber and transfer immediately to the IVIS (*In Vivo* Imaging System) with continuous anesthesia during measurement (1.5%). Use an untreated mouse (PBS) as control.

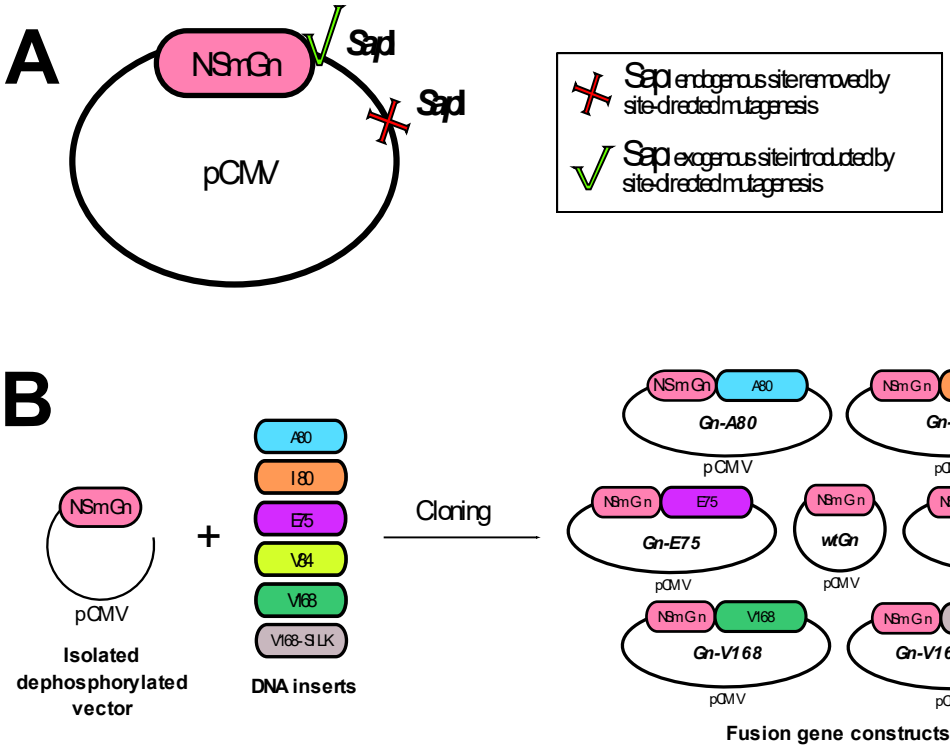
3. Scan the animals for fluorescence at excitation and emission wavelengths of 470 and 800 nm, respectively. Fluorescence of animals is plotted by subtracting the background from the untreated (PBS) mouse.

Viral glycoproteins are responsible for the interactions between the infectious agent and host cells that are able to recognize the antigen. Consequently, both Gn and Gc

glycoproteins constitute major targets for neutralizing antibodies created by the immune system and possess a hydrophilic nature [24]. For this reason, the gene library includes three different fusion genes (Gn-A80, Gn-I80 and Gn-V84) based on hydrophobic residues, namely alanine, isoleucine and valine. These three designs have previously been shown to self-assemble at physiological temperature [25, 26]. Thus, these designs lead to the formation of amphiphilic structures comprising an ELR core covered by the hydrophilic Gn glycoprotein.

The Gn-E75 construct includes a glutamic acid-rich ELR block in the design. The chimeric protein resulting from this fusion gene has a markedly hydrophilic nature and is not able to self-assemble under physiological conditions, thereby remaining in a soluble state [27]. Thus, these four constructs allow us to compare the immunogenic potential of amphiphilic aggregates and soluble glycoproteins with a higher hydrophilicity than in nature. However, the molecular weight of the Gn glycoprotein (73.4 kDa) is much higher than that of the ELR blocks used in these constructs (30.6, 31.3, 34.0 and 34.5 kDa for A80, E75, I80 and V84, respectively; Figure 3). To determine how important this balance between the antigen and the adjuvant is, a new construct was constructed to have a similar weight for the ELR block and the viral antigen in the nanovaccine. Thus, the V168 block (69 kDa) was recombinantly fused to Gn glycoprotein (73.4 kDa). Apart from a more balanced design, the reinforced polymeric component may lead to an easier aggregation state in a physiological environment due to the direct effect of ELR chain length on the ability to self-assemble [26, 28]. Finally, Gn-V168-SILK includes the $[V(GAGAGS)_5G]_2$ sequence from silk fibroin to enhance the aggregation stability (Figure 3), since the silk fibroin from

silkworm is characterized by an irreversible self-assembly into amphiphilic β-sheet secondary structures [29].


A
NSmGn
SapI
SapI
pCMV
SapI endogenous site removed by site-directed mutagenesis
SapI exogenous site introduced by site-directed mutagenesis
B
NSmGn
pCMV
Isolated dephosphorylated vector
+
A80
I80
E75
V84
V168
V168-SILK
DNA inserts
Cloning
NSmGn A80
Gn-A80
pCMV
NSmGn I80
Gn-I80
pCMV
NSmGn E75
Gn-E75
pCMV
NSmGn
wtGn
pCMV
NSmGn V84
Gn-V84
pCMV
NSmGn V168
Gn-V168
pCMV
NSmGn V168-SILK
Gn-V168-SILK
pCMV
Fusion gene constructs
C
wtGn
NSmGn
Gn-A80
NSmGn
(VPAVG)80
Gn-I80
NSmGn
(VGIPG)80
Gn-E75
NSmGn
[(VPGVG)2 VPGEG (VPGVG)2]15
Gn-V84
NSmGn
(VPGVG)84
Gn-V168
NSmGn
(VPGVG)168
Gn-V168-SILK
NSmGn
(VPGVG)168 [(GAGAGS)5]2
D
NSmGn
DNA insert
DNA vaccine
pCMV
In vitro biosynthesis
In vivo immunogenicity
Confocal microscopy
BHK-21 [C-13] cells
TCID50 assay
BALB/c mice
log10TCID50
wtGn
Gn-V84
Gn-E75
Gn-V168
Gn-V168-SILK
Infected

4.

Figure 3. A. Site-directed mutagenesis of the cloning vector pCMV together with the gene insert encoding the NSmGn fusion protein, which results in the removal or introduction of the *Sap*I enzyme restriction sites indicated.
B. Fusion gene constructs obtained from cloning the dephosphorylated vector pCMV-NSmGn and the gene inserts encoding the ELR blocks.
C. Design and composition of the fusion proteins resulting from the fusion genes developed. The ELR blocks include their amino acid composition. Scaled scheme. The molecular weights are as followed: NSmGn, 73.4 kDa; A80, 30.6 kDa; I80, 34.0 kDa; E75, 31.3 kDa; V84, 34.5kDa; V168, 69 kDa; V168-SILK, 73 kDa.
D. Results obtained after *in vitro* and *in vivo* experiments with DNA vaccines. *In vitro* biosynthesis was performed in BHK-21 [C-13] cells, while *in vitro* immunogenicity was tested in BALB/c mice.

5. Notes

1. Different hydrophobic blocks have been reported in the literature, for example tyrosine or alanine instead of isoleucine [13]. A higher hydrophobic content leads to better higher adjuvant properties [26].
2. Although the RDL method based on *SapI* and *EarI* is used in this chapter, there are possible variants, such as concatemerization, overlap elongation polymerase chain reaction (OEPCR), overlap-extension rolling circle amplification (OERCA), or recursive directional ligation by plasmid reconstruction, in which two halves of a parent plasmid, each containing a copy of an oligomer, are ligated together, thereby dimerizing the oligomer and reconstituting a functional plasmid [10, 30]. RDL can also be performed with different enzymes provided they have compatible ends.
3. Cloning plasmids are engineered to simplify their structure and leave only the essential features for high-copy cloning. Their length is usually reduced and they only contain an origin of replication, a drug resistance gene and a multiple cloning site (when the exogenous DNA is for insertion).
4. The percentage of agarose used depends on the size of the fragments to be separated.

Fragment size (bp)	**Agarose (%)**
1000-23000	0.6
800-10000	0.8

400-8000	1
300-7000	1.2
200-4000	1.5
100-200	2

Agaroses with different melting points are available on the market for selected range sizes of DNA fragments.

5. Some phosphatases require a metal cofactor such as Zn^{2+}, which makes them incompatible with common restriction enzyme buffers. In this case, we recommend performing dephosphorylation after DNA purification. If the buffers are compatible, dephosphorylation can be performed simultaneously with DNA digestion or before purifying the dephosphorylated DNA.
6. DNA loading buffer usually contains SDS and EDTA, which stop the dephosphorylation reaction, and is useful for phosphatases that do not heat-inactivate.
7. When performing an analytical electrophoresis, either TAE (tris-acetate-EDTA) or TBE (tris-borate-EDTA) buffer can be used. TBE provides higher resolution of the smallest bands in high agarose percentage gels. However, TBE is not recommended when the DNA fragment is required for further purification and ligation steps because TBE gels afford poor gel recovery and borate ions are reported to inhibit DNA ligase. We recommend performing all the protocol in TAE buffer to avoid undesired mistakes.
8. The voltage applied depends on the cuvette dimensions and is usually set at 3-10 V/cm distance between the electrodes. For preparative electrophoresis we recommend the minimum voltage to obtain more defined bands. The run time depends on the size of the fragments, the percentage of agarose and the voltage applied. The electrophoresis is usually run until the band of interest has migrated 40–60% of the gel length.
9. Due to the low yield of the purification, we recommend measuring the plasmid concentration in a micro-volume UV-VIS Spectrophotometer to avoid loss of sample.
10. Most of the commercial strains of *Escherichia coli* can currently be used depending on the specific cloning requirements, such as high-quality plasmid preparations, blue/white screening, fast colony growth, methylation-free plasmid etc. XL1-Blue, DH5alfa and JM109 are some of the most widely used strains.
11. Although Star Activity should not be observed when *EarI* is used according to the protocol, we have noticed problems of over-digestion when this reaction is run overnight and thus recommend not to exceed 5-6 hours of digestion.
12. A 1:5 molar ratio of plasmid to insert is a good starting point, but this ratio can be changed depending on the size of the insert with respect to the plasmid. Shorter inserts generally need a higher ratio.
13. Transformation efficiencies will be approximately 10-fold lower for reaction ligations than for a complete plasmid
14. Expression plasmids contain features to maximize gene expression, such as a strong promoter, a strong termination codon, a transcription termination sequence

and a strong translation initiation sequence. Although different expression vectors are available, pET vectors, which work under the control of the T7 lac promoter and are induced by lactose, provide the highest level of protein expression.

15. It is important to consider the characteristics of both gene and protein before choosing the *E. coli* expression strain to use. The use of *E. coli* strains BL21(DE3) and BLR(DE3) allows the recombinant protein transcription to be closely regulated. Indeed, the expression process is under control of the bacteriophage T7 promoter, which is only recognized by the T7 RNA polymerase after induction. These strains reduce expression of potential toxic peptides by lowering the background expression. Moreover, BLR(DE3) strains lacking recombinase systems (i.e. RecA-) stabilize target genes, which is an especially important characteristic for genes containing repetitive sequences. The deficiency of some proteases (lon and OmpT) increased expressed protein stability when required. Additionally, other commercial *E. coli* strains are available for various protein expression requirements, such as Origame BTM strains for expressions of proteins which require a folding with disulfide bonds, or Artic-ExpressTM, which is used in protein expression processes at low temperatures.
16. Several commercial competent bacterial cells are available; in these cases, follow the protocol provided by the manufacturer. However, it is also possible to obtain competent cells in your own laboratory following processes like the TSS method. This method is based on two reagents (polyethylene glycol (PEG), and dimethyl sulfoxide or (DMSO)), which are able to permeabilize the bacterial wall and membrane, thus resulting in a suitable transformation efficiency. 1x TSS (Transformation and Storing Solution) comprises LB broth with 10% (w/v) PEG, 5% (v/v) DMSO and 50 mM $MgCl_2$ at (pH 6.5). The mixture can be prepared without DMSO and autoclaved, subsequently adding the DMSO. Another option is to prepare the complete mixture and sterilize by filtration. Mix the cells with 2x TSS in a 1:1 volume ratio to achieve a 1x TSS final concentration and have your cells ready for use immediately. Then, transform the cells with the plasmid DNA of interest [31].
17. The auto-inducible modified TB medium (mTB) is Terrific Broth (TB) supplemented with lactose, glucose, glycerol and salts. Glucose and glycerol can support bacterial culture growth during the first phase equally as effectively, thus doing away with the need for other carbon sources. In this phase, the biomass increases until it starts utilizing lactose as carbon source, which induces recombinant protein expression. This self-induction is due to lactose in the medium and its use allows higher final yields in the expression of recombinant proteins [32].
18. The most common antibiotics employed are used at the following final concentrations: Carbenicillin (disodium salt) 50 μg/mL, Ampicillin (sodium salt) 50 μg/mL, Chloramphenicol 34 μg/mL, Kanamycin (sulfate) 30 μg/mL, Tetracycline 12.5 μg/mL and Streptomycin 25 μg/mL.
19. 1X Phosphate-buffered saline (1X PBS) is an isotonic solution that is used as wash buffer and contains 37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.

20. The washing steps for the bacterial culture prior to SDS-PAGE are short and are performed before cell rupture and denaturalization of the macromolecules by boiling in the presence of loading buffer. Washing can also be performed with deionized water without compromising the result. These washes need not preserve the osmotic balance with salt because cells will be ruptured in subsequent steps.
21. To prepare protein loading buffer with a 5X concentration, prepare a 1M pH 6.5 Tris-HCl solution in ultrapure water type I. Add 10% (w/v) SDS, 50% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 2% (w/v) Bromophenol Blue (BPB)t gove a final concentration of 312.5 mM Tris in the 5X solution. 2-Mercaptoethanol can be substituted by 500 mM DTT in the protein loading buffer composition. SDS interacts with positively charged amino acids in proteins, thereby disrupting the structural interactions of the proteins to separate them on the basis of their size. Moreover, a reducing agent such as 2-mercaptoethanol or DTT is present to reduce disulfide bonds.
22. To analyze high- and low-molecular weight bands on the same gel, develop a gradient gel. Acrylamide total percentage (%T) affects the protein migration, therefore it is especially important to select the gel type that offers optimum resolution of your sample. For example, use 10% T gel to separate proteins with a weight of 90-100 kDa, whereas 12% T gels are more appropriate for proteins of less than 60 kDa. Nevertheless, if you are interested in analysing smaller peptides, a Tricine SDS-PAGE should be carried out (Bio-Rad Laboratories, I. A Guide to Polyacrylamide Gel Electrophoresis and Detection).
23. It is possible to preserve the clones of interest by making a long-term stock. One of most widely used and practical methods is the production of a glycerol stock. Bacteria can be stored for years at -80C in a high percentage of glycerol. Thus, the selected colony is grown on LB with 0.5% (v/v) glucose at 37°C with shaking (250 rpm). When the culture is in the exponential growth phase (optical density at 600 nm (OD600) of 0.6-0.8), grown bacteria are transferred to a cryovial and 0.2 volumes of 80% (v/v) sterile glycerol are added. Due to the high viscosity of glycerol, it is recommended to use dilute solutions. The solution is obtained by diluting 100% glycerol in deionized water. A final concentration of 15-25% (v/v) glycerol is required.
24. Addition of a carbon source, such as glucose, to Luria-Bertani medium (LB medium) avoids the need to use amino acids as carbon source for cell metabolism when the cell volume decreases [33].
25. The production process can be scaled up using a bioreactor. This method allows a reduction in process time as well as greater control of the dissolved oxygen concentration (and IPTG, when needed), controlled bacterial growth and steady pH, in addition to being a scalable and high-performance process. The use of bioreactors guarantees OD_{600} values greater than 10 [34].
26. The inoculated volume must not be higher than 1/30 of the final volume culture.
27. All flasks used must be sterilized previously. Furthermore, to boost the aeration of the culture and its level of dissolved oxygen, the final volume of the flask should be four times higher than the culture volume in it. When oxygen dissolved in the broth is limited, bacterial growth is slower and recombinant protein production is poor. Moreover, oxygen limitation induces the expression of

anaerobic response genes and anaerobic metabolites, thus leading to spoilage of the culture medium and a lower yield of the protein of interest [35].

28. In our experience, the best way to determine the correct moment to stop production is to take samples at different times and measure their absorbance. The process should be stopped when the absorbance value has stabilized (stationary phase), before beginning to decline (death phase). The blank should be the supernatant after centrifuging one sample at 13,400 g for 45 s. The optical density values obtained at 600 nm (OD_{600}) will rely on the production method. For example, if flasks are used it is possible to obtain OD_{600} values of up to 10, whereas with a bioreactor OD_{600} value higher than 10 are routinely obtained.
29. TBS wash buffer comprises 20 mM Tris-base and 140 mM NaCl pH 7.6. This buffer must contain salt to maintain the osmotic balance and not damage bacteria. In addition, to obtain a higher yield, it is possible to harvest the cells by centrifuging all culture volume in the same containers over several steps.
30. TE sonication buffer comprises 10mM Tris-base and 1 mM EDTA pH 8. Ethylenediaminetetraacetic acid (EDTA) is a chemical chelating agent that removes contaminating divalent magnesium cations, a membrane-stabilizing ion, thereby reducing the activity of those proteases that need magnesium ions to function [36].
31. To avoid local overheating and consequent protein denaturation, maintain the sample at 0-4°C. To keep samples cold, it is possible to place a chiller inside the disruptor or to place them in ice for 5 min after each pulse.
32. If purification is not to be started immediately, these phases can be stored at -20°C for a long period. When ready for purification, allow the samples to thaw slowly at 0-4°C the day before.
33. Depending on the ITC parameters and residue composition of the ELR, the supernatant salt concentration and possible pH changes must be evaluated. The salt usually used in this case is NaCl. Indeed, it is possible to decrease the ELR transition temperature by increasing the salt concentration. Because a higher temperature could denature some proteins, this method is preferred [37].
34. Each specific polymer requires a different number of ITC cycles. Some proteins can be achieved in high purity after only a few cycles, whereas others require more than 5 cycles to be purified effectively. The potential loss of proteins in each cycle should be taken into account, especially with hydrophobic ones, which tend to be lost in the cell lysate [38].
35. It is possible to perform post-translational chemical modifications to increase the immunogenic activity of the protein. One example of these modifications is chemical glycosylation by carbohydrate chemical activation, PEGylation or acylation [39, 40].
36. Only plasmid DNA isolated from dam+ *E. coli* strains is suitable for this mutagenesis protocol as it is methylated.
37. To ensure successful amplification, the concentration of dsDNA template can be set at between 5 and 50 ng while keeping the primer concentration constant. In this case, 50 ng of dsDNA template is used.
38. The A260 value should be within the instrument's linear range (0.1–1.0). Good-quality DNA will have an A260/A280 ratio of 1.7–2.0. Lower and higher lower ratios indicate the presence of contaminants, such as RNA or proteins. Strong

absorbance at around 230 nm indicates that organic compounds or chaotropic salts are present in the purified DNA. The A260/A230 ratio indicates the amount of salt in the purified DNA. The A260/A230 ratio should be greater than 1.5. Lower ratios indicate higher amounts of salt.

39. Endotoxin-free DNA improves transfection into eukaryotic cells, therefore endotoxin-free plastic tips and tubes must be used for elution.
40. High-quality DNA is critical for successful transfection. 260/280 nm ratios > 1.8 are recommended for high transfection efficiency.
41. Do not wash between the blocking step with FBS 2% and incubation with the RVFV-immunized mouse serum that is used after 1:100 dilution in PBS-2% FBS. Incubation with RVFV-immunized mouse serum at 37°C improves the fluorescence signal compared to incubation at room temperature.

1= (https://ec.europa.eu/info/live-work-travel-eu/coronavirus-response/public-health/eu-vaccines-strategy_en.)

[1] E.V. Strategy.
[2] B. Sun, X. Zhao, Y. Wu, P. Cao, F. Movahedi, J. Liu, J. Wang, Z.P. Xu, W. Gu, ACS applied materials & interfaces, 13 (2021) 14015-14027.
[3] C. García-Arévalo, J.F. Bermejo-Martín, L. Rico, V. Iglesias, L. Martín, J.C. Rodríguez-Cabello, F.J. Arias, Molecular pharmaceutics, 10 (2013) 586-597.
[4] J. Gonzalez-Valdivieso, B. Borrego, A. Girotti, S. Moreno, A. Brun, J.F. Bermejo-Martin, F.J. Arias, Molecular pharmaceutics, 17 (2020) 1608-1620.
[5] J.B. Ulmer, J.J. Donnelly, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Deck, C.M. DeWitt, A. Friedman, et al., Science, 259 (1993) 1745-1749.
[6] D.W. Urry, T. Hugel, M. Seitz, H.E. Gaub, L. Sheiba, J. Dea, J. Xu, T. Parker, Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 357 (2002) 169-184.
[7] K.M. Ruff, S. Roberts, A. Chilkoti, R.V. Pappu, J Mol Biol, 430 (2018) 4619-4635.
[8] J. Rodríguez-Cabello, A. Girotti, A. Ribeiro, F. Arias, Synthesis of Genetically Engineered Protein Polymers (Recombinamers) as an Example of Advanced Self-Assembled Smart Materials, in: M. Navarro, J.A. Planell (Eds.) Nanotechnology in Regenerative Medicine, Humana Press2012, pp. 17-38.
[9] J.K. Deyling, E.M. Turk, N.B. Holland, Methods in molecular biology, 1798 (2018) 265-275.
[10] A. Girotti, A. Fernandez-Colino, I.M. Lopez, J.C. Rodriguez-Cabello, F.J. Arias, Biotechnol J, 6 (2011) 1174-1186.
[11] Y. Li, Y. Wang, J. Cheng, X. Zhou, H. Lu, X. Zhang, X. Xia, H. Sun, Virol J, 17 (2020) 72.
[12] Y. Li, Y. Lin, G. Xin, X. Zhou, H. Lu, X. Zhang, X. Xia, H. Sun, Vaccine, 38 (2020) 3952-3959.
[13] R.S. Ingrole, W. Tao, J.N. Tripathy, H.S. Gill, Nano LIFE, 4 (2014) 1450004.
[14] D.M. Floss, M. Mockey, G. Zanello, D. Brosson, M. Diogon, R. Frutos, T. Bruel, V. Rodrigues, E. Garzon, C. Chevaleyre, M. Berri, H. Salmon, U. Conrad, L. Dedieu, J Biomed Biotechnol, 2010 (2010) 274346.
[15] L. Duvenage, Hitzeroth, II, A.E. Meyers, E.P. Rybicki, J Virol Methods, 191 (2013) 55-62.
[16] S. Cho, S. Dong, K.N. Parent, M. Chen, Journal of drug targeting, 24 (2016) 328-339.
[17] P. Wang, S. Dong, P. Zhao, X. He, M. Chen, Biomaterials, 182 (2018) 92-103.
[18] Y. Li, M. Fujita, D. Boraschi, Frontiers in immunology, 8 (2017) 472.

[19] D. Kim, Y. Wu, Y.B. Kim, Y.K. Oh, Drug delivery and translational research, (2021).
[20] J.E. Kwak, Y.I. Kim, S.J. Park, M.A. Yu, H.I. Kwon, S. Eo, T.S. Kim, J. Seok, W.S. Choi, J.H. Jeong, H. Lee, Y. Cho, J.A. Kwon, M. Jeong, J.N. Maslow, Y.E. Kim, H. Jeon, K.K. Kim, E.C. Shin, M.S. Song, J.U. Jung, Y.K. Choi, S.H. Park, Nature communications, 10 (2019) 3836.
[21] A. Brun, Methods in molecular biology, 1349 (2016) 1-24.
[22] K.J. Linthicum, S.C. Britch, A. Anyamba, Annual review of entomology, 61 (2016) 395-415.
[23] C.R.S. McEntire, K.W. Song, R.P. McInnis, J.Y. Rhee, M. Young, E. Williams, L.L. Wibecan, N. Nolan, A.M. Nagy, J. Gluckstein, S.S. Mukerji, F.J. Mateen, Frontiers in neurology, 12 (2021) 634827.
[24] M. Pepin, M. Bouloy, B.H. Bird, A. Kemp, J. Paweska, Veterinary research, 41 (2010) 61.
[25] C. García-Arévalo, J.F. Bermejo-Martín, L. Rico, V. Iglesias, L. Martín, J.C. Rodríguez-Cabello, F.J. Arias, Molecular pharmaceutics, 10 (2013) 586-597.
[26] A. Ribeiro, F.J. Arias, J. Reguera, M. Alonso, J.C. Rodriguez-Cabello, Biophys J, 97 (2009) 312-320.
[27] D.W. Urry, J Protein Chem, 7 (1988) 1-34.
[28] A. Girotti, J. Reguera, F.J. Arias, M. Alonso, A.M. Testera, J.C. Rodríguez-Cabello, Macromolecules, 37 (2004) 3396-3400.
[29] T. Asakura, J. Yao, T. Yamane, K. Umemura, A.S. Ulrich, Journal of the American Chemical Society, 124 (2002) 8794-8795.
[30] J.R. McDaniel, J.A. Mackay, F.G. Quiroz, A. Chilkoti, Biomacromolecules, 11 (2010) 944-952.
[31] C.T. Chung, S.L. Niemela, M.R. H., Proceedings of the National Academy of Sciences of the United States of America, 86 (1989) 2172-2175.
[32] F.W. Studier, Protein Expr Purif, 41 (2005) 207-234.
[33] G. Sezonov, D. Joseleau-Petit, R. D'Ari, J Bacteriol, 189 (2007) 8746-8749.
[34] T. Collins, M. Barroca, F. Branca, J. Padrao, R. Machado, M. Casal, Biomacromolecules, 15 (2014) 2701-2708.
[35] M. Krause, K. Ukkonen, T. Haataja, M. Ruottinen, T. Glumoff, A. Neubauer, P. Neubauer, A. Vasala, Microb Cell Fact, 9 (2010) 11.
[36] A. Monico, E. Martinez-Senra, F.J. Canada, S. Zorrilla, D. Perez-Sala, PloS one, 12 (2017) e0169843.
[37] J. Reguera, D.W. Urry, T.M. Parker, D.T. McPherson, J.C. Rodriguez-Cabello, Biomacromolecules, 8 (2007) 354-358.
[38] W. Hassouneh, T. Christensen, A. Chilkoti, Curr Protoc Protein Sci, Chapter 6 (2010) Unit 6 11.
[39] C. Temporini, T. Bavaro, S. Tengattini, I. Serra, G. Marrubini, E. Calleri, F. Fasanella, L. Piubelli, F. Marinelli, L. Pollegioni, G. Speranza, G. Massolini, M. Terreni, Journal of chromatography. A, 1367 (2014) 57-67.
[40] T. Bavaro, S. Tengattini, L. Piubelli, F. Mangione, R. Bernardini, V. Monzillo, S. Calarota, P. Marone, M. Amicosante, L. Pollegioni, C. Temporini, M. Terreni, Molecules, 22 (2017).