

A novel lipase-catalyzed method for preparing ELR-based bioconjugates

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ABSTRACT:

Herein we present a novel one-pot method for the chemical modification of elastin-like recombinamers (ELRs) in a mild and efficient manner involving enzymatic catalysis with *Candida antarctica* lipase B. The introduction of different functionalities into such ELRs could open up new possibilities for the development of advanced biomaterials for regenerative medicine and, specifically, for controlled drug delivery given their additional ability to respond to stimuli other than pH or temperature, such as glucose concentration or electromagnetic radiation.

Candida antarctica lipase B immobilized on a macroporous acrylic resin (Novozym 435) was used to enzymatically couple different aminated substrates to a recombinamer containing carboxylic groups along its amino acid chain by way of an amidation reaction. A preliminary study of the kinetics of this amidation in response to different reaction conditions, such as solvent, temperature or reagent ratio, was carried out using a phenylazobenzene derivative (azo-NH₂) as a model. The optimal amidation conditions were used to couple other amine reagents, such as phenylboronic acid (FB-NH₂) or polyethylene glycol (PEG-NH₂), thus allowing us to obtain photoresponsive, glucose-responsive or PEGylated ELRs that could potentially be useful as sensors in devices for controlled drug delivery.

Keywords: Elastin-like recombinamers, enzymatic modification, amidation reaction, CAL-B lipase, polymer bioconjugation, controlled drug delivery.

1. Introduction

Natural materials are intricate and multifunctional structures that often inspire scientists to design novel classes of synthetic materials. This is the case for elastin-like recombinamers (ELRs), a type of recombinant biopolymers with a structure based on elastin, the extracellular elastic protein found in higher animals [1]. As these recombinamers are designed at the gene level, the high precision control in gene construction is transferred onwards into the final polypeptide, thus meaning that their physical and bioactive properties can be finely tuned as desired using the most appropriate amino acid composition for different biomedical and nanotechnological applications [2-4]. The degree of control and complexity of such recombinamers is not practically achievable using more conventional synthetic technologies.

ELRs exhibit self-assembly behaviour and possess a transition temperature (T_t) in an aqueous environment such that, below T_t , the free polymer chains adopt random coil conformations, whereas above it they fold into an organized structure known as a β -spiral. Although ELRs respond primarily to temperature, the effects of other stimuli, such as pH, ionic strength, and concentration, also affect the transition phenomenon [5].

Despite the above, recombinant production only allows us to obtain polypeptides based almost exclusively on natural amino acids. Although that has been sufficient to date for the preparation of, for example, pH- or calcium-sensitive materials [1] there is no natural amino acid that can render light or other types of responsiveness. As such, the design of new and effective ways of modifying these recombinamers are of interest since this would make it possible to introduce suitable functionalities to make them sensitive to other stimuli that can not be accessed by genetic engineering.

One of the recombinamers designed by our group (ELRGlu15) contains equally spaced carboxyl groups from glutamic amino acids along its chain. These functional groups confer pH sensitivity on the recombinamer as well as the ability to undergo derivatization by chemical transformation [6]. Consequently, the initially pH-responsive material (ELRGlu15) can be modified to provide a new material (m-ELRGlu15) which, for example, is responsive to other stimuli of technological or biomedical interest, such as electromagnetic radiation or glucose concentration. Likewise, functionalization allows us to obtain more complex polymer architectures, such as polymer brushes, by grafting synthetic polymer branches onto this recombinamer, thereby combining the functionality of naturally produced and inspired materials and fully synthetic materials.

Despite the advantages of using enzymes in functionalization reactions, few examples of synthetic polymer functionalization involving either natural or synthetic polymers have been reported in the literature [7-10].

Lipases (triacyl glycerol hydrolase, EC 3.1.1.3) are one of the most promising enzymes for broad practical applications in organic synthesis. They are stable and rugged enzymes that are also active with a wide variety of non-natural reagents. In addition, these non-toxic biocatalysts are also renewable, relatively cheap compared with other enzymes and a variety of lipases are becoming commercially available [11].

Due to their high efficiency, recyclability, ability to react under milder conditions and easy separation, immobilized *Candida antarctica* lipase B (CAL-B) assisted functionalization of polymers is of great importance. For example, CAL-B has been shown to catalyze transesterification reactions employing different acyl donor substrates as α -telechelic PEG, hydroxy-terminated polyisobutylene or polystyrene derivatives, ring-opening polymerization (ROP) of carbohydrates giving rise to highly end-functionalized polymers or ester bond formation between the terminal carboxylic acid group of an organosilicon compound and the primary hydroxy group at the C-6 position of (*R*)-ethyl glucoside [8]. However, no studies in which CAL-B or a lipase in general acts upon a protein-like substrate have been found in the literature.

With these antecedents in mind, we planned to conceive an alternative method (simple and direct route) for introducing different groups into the ELR chemical structure that allow an additional sensitivity towards factors such as electromagnetic radiation or sugar concentration in the medium, which could be used for light-triggered drug release at localized delivery sites, for example, in photodynamic therapy and other localized therapies [12], or as a sensor for determining sugar levels[13]. Hydrogels made from such modified ELRs could be used in medicine as intelligent devices, for example, to self-regulate insulin-

delivery systems [14]. Similarly, in order to obtain a much more complex molecular architecture, we decided to graft PEG onto our intrinsically linear macromolecular ELRs as side chains. These branched polymers should combine the smart functionality of ELRs with the inherent functionalities of the branched structures [15].

Herein we describe an enzymatic approach for constructing a series of derivatives (m-ELRGlu15) of this functional polymer containing free γ -carboxylic groups (ELRGlu15), that will subsequently act as acyl donor group substrates. The recombinant protein is enzymatically modified by amidation using a commercial immobilized microbial lipase (Novozyme 435 CAL-B) as biocatalyst. In this case, the acyl acceptor substrates are different nitrogen nucleophiles, namely *p*-phenylazoaniline (azo-NH₂), 4-[(2-amino)carbamoyl]phenylboronic acid (FB-NH₂) and polyethyleneglycol (PEG-NH₂). All reactions were performed under mild conditions, at moderate temperatures, in organic solvents, and in the absence of drying agents in the reaction mixture. The influence of reaction temperature, solvent, time, weight percentage (%) of lipase to biopolymer, and structure of the substrate on the progress of the amidation reactions has been studied. To the best of our knowledge, no amidation of ELRs has been carried out using this type of enzyme.

2. Experimental section

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Co. (Madrid, Spain) and used as received. *Candida antarctica* Lipase B (CALB) immobilized on macroporous acrylic resin (Novozym 435, Lot No. LC200229 (with an activity in propyl laurate units of 11,200 PLU/g) was kindly provided by Novozymes (Bagsvaerd, Denmark). Although this system functions over a wide temperature range (20-110 °C), the typical reaction temperature was 30-60°C.

2.2. ELRGlu15

The ELR was produced by genetically engineered protein biosynthesis in *Escherichia coli*, and was purified using several cycles of temperature-dependent reversible precipitation, as described elsewhere. It exhibits the amino-acid sequence (MESLLP) [(VPGVG)₂(VPGEG)(VPGVG)₂]₁₅ [16].

The final product was characterized by SDS-PAGE, NMR, FTIR spectroscopy, MALDI-ToF mass spectrometry and amino-acid analysis.

2.3. Preparation of 4-[(2-amino)carbamoyl]phenylboronic acid (FB-NH₂)

According to the protocol reported by Kataoka, *et al.* [17], the synthetic route to 4-[(2-aminoethyl)carbamoyl]phenylboronic acid implies the reaction of 4-(chloroformyl)phenylboronic acid with an excess of ethylenediamine.

The first step is the reaction of 4-carboxyphenylboronic acid (CPBA; 10 g, 60.3 mmol) with thionyl chloride (150 mL, 2.1 mol) under an argon atmosphere. This suspension was stirred and refluxed at 88 °C for 24 h to give 4-(chloroformyl)phenylboronic acid. After evaporation of the remaining thionyl chloride, the

flask was charged with argon again. The contents of the flask [4-(chloroformyl)phenylboronic acid] were then suspended in 60 mL of distilled THF. The cooled suspension was then slowly added dropwise to distilled ethylenediamine (200 mL, 3.0 mol) in the presence of distilled triethylamine (10 mL, 71.9 mmol) in an ice bath under an argon atmosphere. After stirring the mixture for 20 h, the unreacted ethylenediamine was evaporated and the residue was dissolved in 100 mL of distilled water. The pH of the solution was then adjusted to ca. 4 by addition of 1 N HCl. A white precipitate, assigned from the ^1H NMR spectrum to be mainly the 2:1 reaction product of 4-(chloroformyl)phenylboronic acid with ethylenediamine [4,4'-(ethylenedicarbamoyl)phenylboronic acid], was filtered off. The filtrate was concentrated and then stored at 4°C overnight to give a white crystalline product. These crystals of 4-[(2-aminoethyl)carbamoyl]phenylboronic acid were dissolved in water and recrystallized twice. Yield: 4.94 g (49% based on CPBA). ^1H NMR (400 MHz, D_2O) δ : 3.3 [$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-}$, 2H], 3.7 [$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-}$, 2H], 7.8 [$\text{-NH-COC}_6\text{H}_4\text{-B(OH)}_2$, 4H].

2.4. Preparation of *m*-ELRGlu15

In a typical amidation reaction, the amine reagent (5 mmol, 10 eq) was treated with ELRGlu15 (1 g, 0.033 mmol ELR, 0.50 mmol COOH, 1eq) in the corresponding organic solvent (50 mL) in the presence of 100 mg of Novozym 435 (CAL-B) (10 wt % relative to the total weight of the recombinamer). The 10 equivalents of amine group were calculated taking into account the presence of 15 glutamic acids in the ELR. The photoresponsive ELRs were synthesized using *p*-phenylazoaniline (azo- NH_2) and the glucose-sensitive ELR was synthesized using 4-[(2-aminoethyl)carbamoyl]phenylboronic acid (FB- NH_2) as amine reagent. Likewise, the PEGylated ELR was obtained by treatment with *O*'-(2-aminoethyl)-*O*'-methylpoly(ethylene glycol) (PEG- NH_2).

The reaction mixture was kept under orbital agitation (at 300 rpm) for 1-7 days and at 25 or 40 °C, depending on the assay. After this time, the enzyme was removed by filtration using a fritted-glass filter (medium porosity) and the substrate-functionalized polymer was precipitated into ether after filtering off the enzyme, washed with additional diethyl ether fractions and dried in a vacuum oven at room temperature for 24 h. Once the *m*-ELRs had been dried, they were solubilized in cold water and the crude products were purified by exhaustive dialysis against water. A white (for PEG and FB) or yellow product (for *p*-phenylazoaniline) was obtained after lyophilization. Chemical yields were in the range 70-85%. All experiments were conducted in triplicate and the reproducibility was found to be less than $\pm 5\%$

In order to study the kinetics of the amidation, taking the reaction between *p*-phenylazoaniline (azo- NH_2) and the free glutamic γ -carboxylic group of ELRGlu15 as a reference, a first series of experiments (n=3) was carried out in a 100 ml Erlenmeyer flask using 2:1 DMF:THF as solvent (10 ml), at 40 °C, and amine group to γ -carboxyl residue ratios of 1:1, 5:1 and 10:1, with the same amount of lipase each time (100 mg, 10 wt %). Another series of experiments (n=3) was carried out using different amounts of lipase (5, 10 and 20 wt %) for a 5:1 molar ratio of amine group to γ -carboxyl residue and in the same mixture of solvents. Reactions were carried out simultaneously in quadruplicate, stopping each of them at different times in order to analyse the effect of reaction time,

amine:carboxyl ratio and CALB weight % on conversion. Control experiments in which no lipase was present were also carried out.

Further tests at different temperatures (25 and 40 °C) and with different solvents and solvent mixtures were conducted using an optimal ratio of amine group and CAL-B wt %. Finally, the optimal conditions determined above were used to carry out coupling reactions between ELRGlu15 and other reagents such as 4-[(2-aminoethyl)carbamoyl]phenylboronic acid (FB-NH₂) and *O*-(2-aminoethyl)-*O*'-methylpoly(ethylene glycol) (PEG-NH₂).

2.5. Instrumental methods

2.5.1. UV-Vis spectroscopy

This technique was carried out using a Cary 50 spectrophotometer (Agilent Technologies, California, USA)

equipped with a temperature-controlled cell holder. Sample organic solutions prepared for spectroscopic analysis had a concentration of around 0.60 mg/mL.

The photochromic coupling ratio was determined by azo group absorbance measurement using a solution of the corresponding m-Azo-ELR in hexafluoropropanol. It was calculated using the Beer-Lambert law, assuming a molar extinction coefficient or molar absorption coefficient (ϵ) of 23000 M⁻¹ cm⁻¹ at $\lambda=340$ nm for the azo group.

The phenylboronic coupling ratio was determined using the 234 nm absorbance. Aqueous mixtures of 4-[(2-aminoethyl)carbamoyl]phenylboronic acid at different concentrations with a constant concentration of biopolymer were used to obtain the corresponding calibration curve ($r=0.996$).

2.5.2. ¹H and ¹³C NMR Spectroscopy

¹H and ¹³C NMR spectra were recorded using a Bruker ARX300 spectrometer (¹H, 300.13 MHz; ¹³C, 75.48 MHz) and Varian unity plus 600 MHz spectrometer (¹H, 599.64 MHz; ¹³C, 150.8 MHz) from Bruker, Massachusetts, USA and Agilent Technologies, California, USA respectively. All measurements were carried out at 298 K with samples of 20–30 mg of the modified elastin like recombinamers, purified as described below and dissolved in 650 μ L of DMSO-d₆. Chemical shifts (δ) are given in ppm.

2.5.3. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was conducted using a Bruker FTIR spectrophotometer (Bruker, Massachusetts, USA). For each spectrum, a 128 scan interferogram was collected in single-beam absorption mode with a 2 cm⁻¹ resolution and a 1 cm⁻¹ interval in the region 4000 to 600 cm⁻¹. FTIR absorption spectra were collected for each sample, and five measurements were averaged to obtain the final FTIR absorption spectrum of the sample. Residual water vapour absorption was interactively subtracted from the sample spectra.

Spectral calculations were performed using OPUS (version 4.2) software (Bruker Biosciences Espanola S.A., Madrid, Spain).

2.5.4. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF)

Molecular weights were determined by matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-ToF), which was performed using a PE Biosystems Voyager-

DE Instrument (Applied Biosystem, California, USA) equipped with a nitrogen laser (337 nm) operating in the positive ion mode with delayed extraction. The ELR samples for MALDI-ToF measurements were prepared in an aqueous 50% (v/v) acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid, using sinapinic acid matrix.

2.5.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (mini VE vertical electrophoresis system from Amersham Biosciences Corp., Bath, UK) with copper staining was performed to assess m-ELRGlu15 purity and molecular weight. A polyacrylamide gel was loaded with 5 μ L of 1 mg.mL⁻¹ m-ELRGlu15 solution.

2.5.6. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) experiments were performed using a Mettler Toledo 822° DSC (Greifensee, Schweiz) with a liquid nitrogen cooler accessory.

Both temperature and enthalpy were calibrated against an indium standard under the same experimental conditions as used for the studied materials. Aqueous solutions of m-ELRGlu15s (50 mg mL⁻¹) were prepared at different pH values. In a typical DSC run, 20 μ L of the solution was placed inside a standard 40 μ L aluminium pan and hermetically sealed. The same volume of water was placed in the reference pan. All samples were equilibrated for 10 min at 0 °C inside the sample chamber before beginning each experiment, and then heated from 0 to 70 °C at a heating rate of 5 °C/min. Scans were run under a nitrogen atmosphere. The transition temperature (Tt) of m-ELRGlu15s in the range 0-70 °C were recorded using the endotherm corresponding to the last step.

3. Results and Discussion

ELRs are biomaterials of high interest because of their applications [18-20] in fields such as tissue engineering, regenerative medicine in general or controlled drug delivery. Moreover, the fact that they can be biosynthesized using the synthetic machinery in bacteria allows us to obtain polymers with a perfectly defined composition and amino acid sequence. The presence in their structure of amino acids such as glutamic acid, that can subsequently be derivatised, allows for modifications at specific locations. In this work, a basic structural composition of ELR was used in order to better analyze the possibility of efficiently carrying out one-pot modifications. The starting ELR used in this study is a pH-sensitive and thermoresponsive ELR that undergoes a characteristic inverse temperature transition (ITT) in which the phase transition can be triggered by a change in pH at the working temperature [16]. The recombinamer ELRGlu15 (MW = 31943 Da) has the amino-acid sequence (MESLLP)[(VPGVG)₂-(VPGEG)-(VPGVG)₂]₁₅. Consequently, carboxylic groups from glutamic acid (E) are uniformly distributed along its chain and these functional groups can undergo amidation. As it is obtained as a recombinamer by genetic engineering, its molecular architecture and stereochemistry are totally controlled. Characterization of this recombinamer is described in greater detail in the supporting information and all of the analyses confirmed the correctness of the biosynthetic and purification processes in terms of purity, sequence and molecular mass.

We must bear in mind that the chemical modification of macromolecules usually involves slow, complex and low yield processes given the complexity of the molecule to be

modified. For this polymer, non enzymatic amidation would require a previous chemical activation of the carboxyl group. As mentioned earlier, in previous studies we have reported chemical modification of ELRGlu15 with *p*-phenylazoaniline (Azo-NH₂) [6] or with 1-(β -hydroxyethyl)-3,3-dimethyl-6'-nitrospiro-(indoline-2,2'[2*H*-1]benzopyran) (Sp-OH) at the free γ -carboxylic acid present in the ELR using the dicyclohexylcarbodiimide coupling method [21]. A moderate photochromic coupling ratio of 17.4% or 45%, as determined by azo or spiroirane group absorbance measurement was obtained even though the reaction was carried out with an excess of reagent.

The need for a new and more efficient way of modifying these ELRs that mitigates the shortcomings of non enzymatic chemical transformation encouraged us to extend our investigations on modification of ELRs, but this time using a simple one-pot transformation and enzymatic catalysis. Thus, we studied modification of the biopolymer ELRGlu15, in a single step, with different amine reagents using *Candida antarctica* lipase B (CAL-B) (see Fig. 1) as this is one of the most active and stable lipases [11]. Moreover, use of this type of enzyme will allow the amidation-based functionalization of a carboxyl-containing elastin-like recombinamer (e.g. ELRGlu15) under anhydrous conditions, thereby preventing hydrolysis [22].

In the present work, the kinetic of amidation of ELRGlu15 with *p*-phenylazoaniline, catalyzed by CAL-B lipase, has been investigated as a model. In order to optimize the amidation process, the effects of various parameters, such as solvent properties, reaction temperature, lipase and substrate concentration, on the coupling reaction ratio were studied. Further modifications with other amine reagents were analysed to confirm the applicability of this new strategy for the modification of macromolecules such as ELRs.

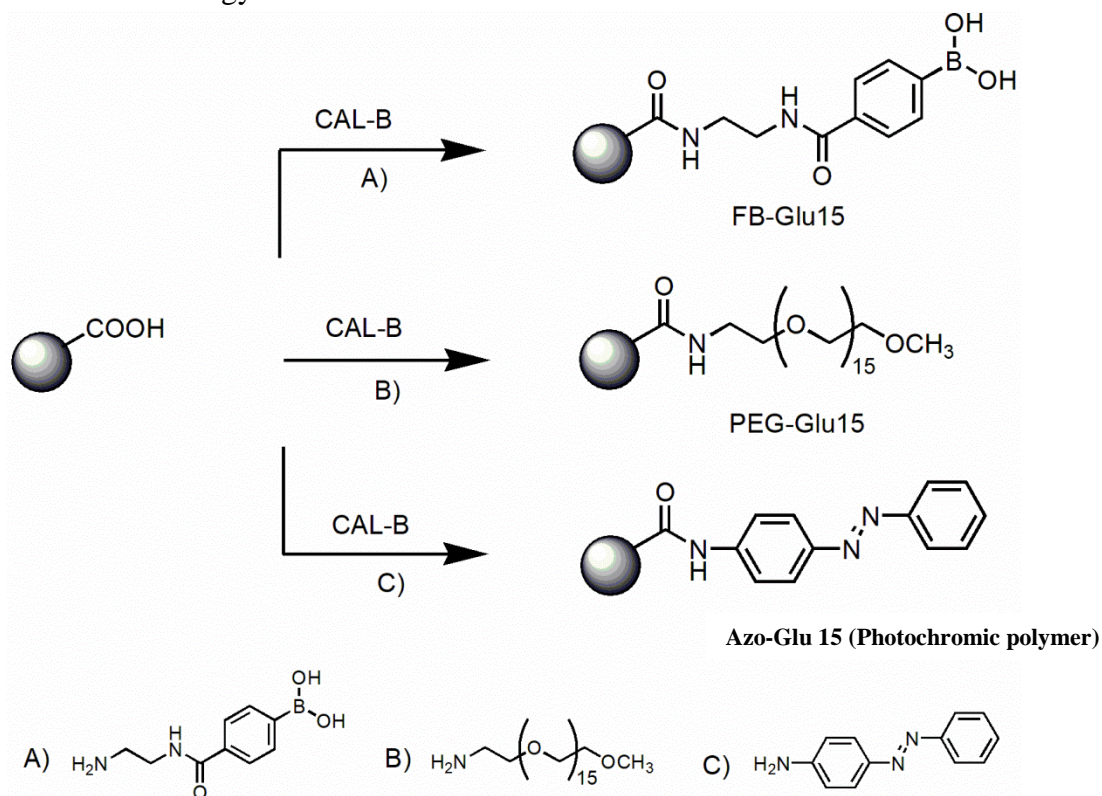


Figure 1. Proposed enzymatic modifications of ELRGlu15 at the free carboxylic group with different amine substrates using CAL-B as catalyst.

A preliminary study was conducted using *p*-phenylazoaniline (azo-NH₂) as nucleophile reagent with the aim of coupling this chromophore in a more efficient manner than when using more conventional amidation chemistry, and in just one step. Once the optimal reaction conditions for this reagent had been established, amidation with other amine reagents was carried out.

After enzymatic reaction and purification, as described in the Materials and Methods section, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) assays were performed in order to assess the degree of recombinamer derivatization from their molecular weight. Recombinamer characterization included NMR spectroscopy, differential scanning calorimetry, and infrared spectroscopy analyses, and the reaction efficiency was also evaluated by way of absorbance measurements (the results are available in the supporting information).

3.1. Reaction Progress with Time.

In order to determine the optimal conditions for the amidation reaction, a kinetic experiment was carried out under different conditions to determine the effect of reaction time on coupling ratio percentage. This allowed us to define the reaction time, ratio of amino to carboxyl groups of the ELR and weight percentage (%) of CAL-B to biopolymer to be used in the experimental design step. Initially, the reaction between the amino group of *p*-phenylazoaniline (azo-NH₂) and the free glutamic γ -carboxylic group of ELRGlu15 was carried out for between 24 hours and 7 days, with a 1:1, 5:1 or 10:1 mol/mol ratio of amino groups to γ -carboxylic group and 10 wt % CAL-B relative to the total weight of the recombinamer in 2:1 DMF:THF as solvent and at 40 °C (Fig. 2A). Once the optimal molar ratio had been established, reactions with 5, 10 or 20 wt % of CAL-B relative to the total weight of the recombinamer (50, 100 and 200 mg, respectively) were carried out under the same conditions (Fig. 2B). Control experiments in which no lipase was present were also performed.

As can be seen from Fig. 2, a similar pattern was observed for all experiments as regards reaction conversion, which increases as a function of reaction time, reaching a maximum after 4 days (96 hours). Extending the reaction time from 4 days to almost 7 days (144 hours) gave approximately the same or a slightly lower ratio. This decrease in coupling ratio may be related to a residual hydrolase activity of the enzyme as the water produced during amidation reaction could act as a competitive nucleophile. Likewise, the lag period observed for *p*-phenylazoaniline amidation is likely due to the availability of hydrophobic reagent substrate and hydrophilic ELR molecules at different phases.

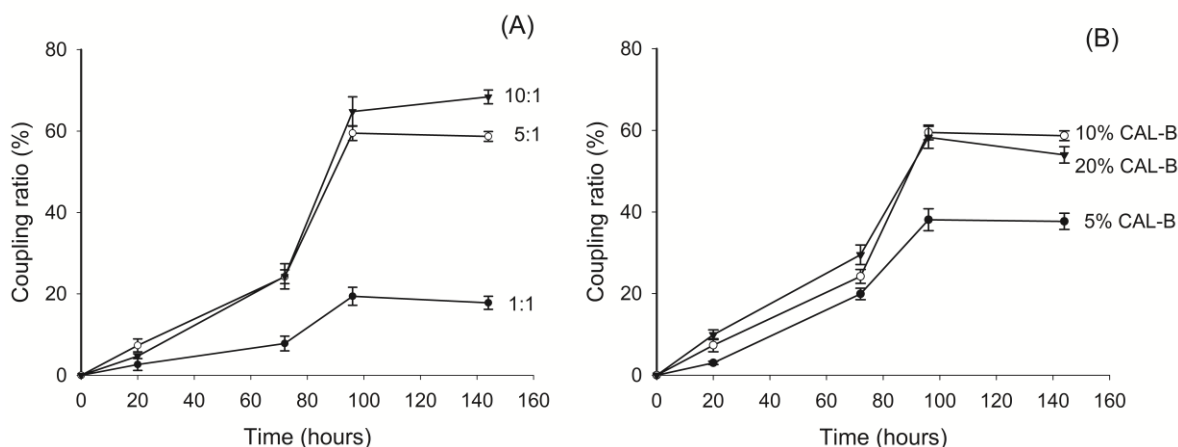


Figure 2. Time course of the enzymatic amidation of ELRGlu15 with azo-NH₂: (A) Influence of NH₂:γ-COOH ratio and (B) influence of enzyme quantity on amidation coupling ratio. Reactions were performed at 40 °C with a 1:1, 5:1 or 10:1 mol/mol ratio of amino groups to glutamic residues (A) or with different lipase concentrations, expressed as mg of catalyst per 100 mg of biopolymer (w/w %) (B). The solvent mixture is 2:1 DMF:THF. A control experiment in which no lipase was present was carried out, with no conversion being detected. All experiments were performed in triplicate.

The first series of experiments with the same 10 wt % of CAL B relative to the total weight of recombinamer (Fig. 2A) showed that the coupling ratio was much higher when working with an excess of reagent but was only slightly higher for a 10:1 than for a 5:1 ratio. As such, we established 5:1 as the optimal mol/mol ratio for amidation.

The influence of lipase concentration on the coupling ratio of the amidation reaction was then studied using a 5:1 molar ratio of *p*-phenylazoaniline amino groups to glutamic residues. As higher coupling ratios were observed for experiments with 10 and 20 wt % CAL B after 4 days, along with slower kinetics when the weight percentage is reduced to 5%, and as a similar behaviour for the two highest wt % has been found up to 4 days, we can conclude that, above 10 wt % CAL B, the amount of enzyme does not influence the coupling reaction ratio. Consequently, this enzyme concentration will be considered to be optimal, with an optimal reaction time of 96 hours.

3.2. Influence of reaction temperature and solvent effect

Once the optimal ELR amidation conditions in terms of weight % lipase (10%), NH₂:free glutamic γ-carboxylic group molar ratio (5:1) and optimal reaction time (4 days) had been established, a study of the reaction of ELRGlu15 with azo-NH₂ under different conditions of solvent and temperature was performed in order to determine the best conditions applicable to other substrates that could be incorporated into the ELR via an enzymatically catalysed amidation reaction. Solvent polarity selection is a crucial factor on enzymatic catalysis in non-aqueous medium, to obtain an adequate coupling ratio due to the direct interference of this on the activity, stability and specificity of the enzyme. In general, the stability of immobilized enzymes increases in organic solvents, thus meaning that they can tolerate higher reaction temperatures respect to reaction with no immobilized enzymes [11]. Similarly, organic hydrophobic solvents are less harmful to enzymes, whereas hydrophilic solvents tend to strip the essential water from the protein structure, thus leading

to loss of enzyme activity. In addition, an ideal organic solvent can help to dissolve the substrate, thus favouring the progress of the reaction without affecting enzymatic activity.

The value of $\log P$, where P is the octanol-water partition coefficient, is generally considered to be a good selection variable for the optimization of biocatalytic systems in organic solvents since it represents the polarity of the solvent. General guidelines for the employment of enzymes in organic solvent usually recommend to use apolar (high $\log P$) solvents to ensure high activity and stability [23]. However, CALB also has a rather unique stability and activity in more polar organic solvents. Indeed, polar solvents are even preferred in some cases due to the increased solubility of hydrophilic reagents [11].

With the above in mind, one of the candidates to perform amidation reaction would be tetrahydrofuran (THF); a low polar solvent, able to maintain the catalytically active conformation of the enzyme and to solubilize amine reagents. As the ELR is not completely dissolved in THF, it must be solubilized in a more polar solvent such as dimethylformamide (DMF) or trifluoroethanol (TFE). Different DMF:THF ratio (1:5, 2:1 and 5:1) will be employed in order to determine the effect of increasing ratios of DMF over the enzymatic activity. On the other hand, to ensure ELR total solubility, a more polar solvent as TFE was also employed (results are shown in Fig. 3). As a control, identical conditions were applied to reactions carried out in the absence of enzyme, with no observable amidation of the substrate being detected.

In order to study the influence of temperature on the amidation process, we performed reactions with the previously established solvents and mixtures of solvents at two different temperatures, 25 °C and 40 °C, in parallel. It is well known that the optimal temperature for an enzymatic reaction is determined by the interaction between the operational stability of the lipase and the process rate [24]. Our findings show that, regardless of the solvent used, the highest amidation activity values for the lipase were obtained at 40 °C (Fig. 3). Similarly, for a given organic solvent, an increase in temperature from 25 °C to 40 °C implies a reduction in the time required to obtain a certain coupling ratio. As can be seen from Fig. 3, the efficiency of the reaction increased threefold when increasing the reaction temperature from 25 to 40°C while keeping all other reaction variables constant (a 16% coupling ratio was observed at 25 °C when using DMF:THF 2:1 as solvent and after 4 days, whereas 56% conversion was observed upon increasing the temperature to 40 °C). The same pattern was observed for the other mixtures of DMF:THF studied. However, when trifluoroethanol was used as solvent, only a slight increase in coupling ratio was observed at the higher temperature of 40°C. Once again, control reactions performed at these temperatures in the absence of the enzyme did not produce any observable amidation of the product.

With respect to the coupling ratio obtained when amidation was carried out in a mixture of DMF:THF, a low conversion was observed at both 25 and 40 °C with a 1:5 ratio, reaching only 20% coupling at the higher temperature after reaction for 4 days. An increase in the DMF proportion to a ratio of 2:1 gave rise to a marked increase in amidation coupling, reaching a value of 56% conversion when the temperature increased to 40 °C. Further addition of DMF (5:1 DMF:THF) had no significant effect on conversion ratio, although it did decrease slightly. These results show that, in this case, a more hydrophilic medium favors the coupling process. The higher enzyme activity at 40 °C in a polar solvent might be due to the better ability to dissolve the ELR, thus increasing the accessibility of the free γ -carboxylic group to the primary alcohol group of serine at the enzyme active site [25].

In order to promote the accessibility of the γ -carboxylic groups to the enzyme, we decided to test TFE as this solvent has previously been used in the chemical modification of ELRGlu15 by way of a carbodiimide-mediated amidation reaction [6]. However, a very low conversion was achieved when performing the reaction in TFE. This result shows that this solvent impedes CAL-B function, probably by promoting various alterations in its water structure, interacting with the protein hydration layer and with the protein structure itself and, finally, by competing with water for enzyme surface interactions. Moreover, the presence of TFE may somehow hinder access of the substrate to the active site, thereby reducing catalytic activity and therefore the coupling reaction.

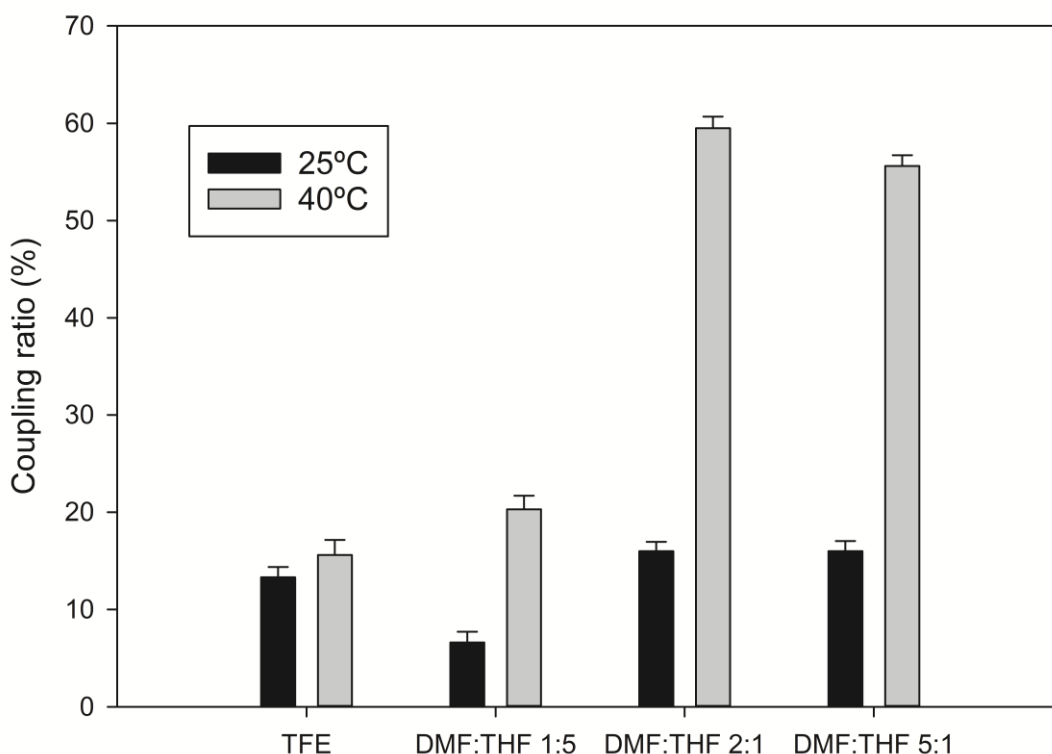


Figure 3. Coupling of phenylazobenzene to the ELR as a function of the temperature and solvent used in the enzymatic reaction after reaction for 4 days. The error bars represent the standard deviation for triplicates. Reaction time: 4 days; ELR:Novozym 435 10:1 (w/w); ratio of amidation agent to carboxyl acid units: 5:1 mol/mol.

In summary, these experiments with the substrate Azo-NH₂ show that the optimal experimental conditions are 40 °C, a reaction time of 4 days and THF:DMF 1:2 as solvent mixture. These reaction conditions were therefore used in subsequent experiments.

3.3. Use of optimal reaction conditions for other enzymatic modifications of the ELR

The optimal reaction conditions for azo coupling (DMF:THF 2:1, 5:1 amine:carboxylic molar ratio, CAL-B 10 wt %, 40 °C and reaction time of four days) determined above were applied to two additional substrates in order to determine whether this methodology can be extended to other enzymatic modifications, thereby efficiently

synthesising ELRs with various end functionalities. Thus, the reaction of ELR-Glu15 with FB-NH₂ and PEG-NH₂, in the presence of CAL-B as catalyst, successfully led to the corresponding modified ELRs, FB-Glu15 and PEG-Glu15, containing phenylboronic and polyethyleneglycol groups, with coupling ratios of 60.8% and 12.8%, respectively.

In the reaction with PEG-NH₂, ELR diblocks with polyethyleneglycol branches were obtained, with two or three branches grafted per macromolecule. In this case, functionalization of ELR-Glu15 did not progress at the same level of conversion as for Azo-NH₂, with approximately nine phenylazobenzene groups being incorporated into the chain in the latter case. However, this is a very promising result since, as far as we are aware, coupling to macromolecules remains an inherently difficult process [8]. Given the structure of the active site in CAL B, steric hindrance in the channel that hosts the amine moiety, which is less spacious than the channel that hosts the acyl moiety, could play an important role [25]. As such, the enzyme exhibits a high degree of selectivity toward amine substrates and, in this case, it is possible that, due to the proximity of the amine group to the bulk of the polymer backbone, the enzyme intermediate has greater difficulty in coordinating to the PEG molecule when generating the new amide bond. Furthermore, coupling with one PEG molecule will prevent coupling with one another.

A very high coupling was obtained in the reaction with FB-NH₂, with an average of nine units FB being incorporated along the ELR chain. The hydrophilic nature of FB-NH₂ and its size, which appears to be a good fit for the CAL-B active centre, likely facilitate the amidation reaction in this case. This new product may allow us to design useful sensor-actuator combinations for drug-delivery systems with interesting applications, for example insulin-delivery systems in patients with diabetes mellitus. The observed dependence of ELR reactivity on the nature of the substrate as a result of steric hindrance at the CAL-B active site has previously been described in the CAL-B-promoted acylation of poly[*N*-(2-hydroxypropyl)-11-ethacryloylaminoundecanamide-co-styrene] copolymers in different compositions [26].

The results obtained with both Azo-NH₂ and FB-NH₂ reveal that a new, simple and effective one-pot methodology has been developed for the amidation of polymers using CAL-B lipase as catalyst when the amine substrates used are small. Moreover, promising results have been obtained for PEG bioconjugation to a macromolecule such as ELRGlu15, both of which are large. As this conjugation promotes an increase in transition temperature (see supporting information), our intention in the future is to modify this mELR, in a second step, in order to provide additional sensitivity to electromagnetic radiation or towards glucose concentration at close to body temperature.

3.4.Azo-Glu15 DSC behaviour vs coupling ratio

Once the amidation reactions had been completed, the chemical structure of the functionalized polymer samples was confirmed by ¹H NMR, ¹³C NMR and FTIR spectroscopy and their purity and molecular weight were determined by performing the corresponding MALDI-ToF and SDS-PAGE assays. The results showed structural changes in the original ELRs as well as the coupling reaction ratio of the different polymers (see Supporting Information).

The thermal transitions of aqueous solutions of the different mELRs synthesized, as determined by differential scanning calorimetry analysis (DSC) at pH 3, showed similar endotherms to that for the starting ELR, thus confirming that the modified biopolymers

conserved their smart nature (see supporting information). The thermograms for these carboxyl-functionalized polymers showed the typical endotherm associated with the ITT. The influence of pH on the ITT (T_t and ΔH) for this set of polymers is of interest. Thus, the γ -carboxylic function of the glutamic acid residue that remains unreacted undergoes strong polarity changes between its protonated and deprotonated states as a consequence of changes in pH around its effective pK_a . At pH 3, for example, complete protonation of all γ -carboxyl residues is considered to have taken place. However, upon increasing the pH, the γ -carboxyl group of the glutamic acid starts to become deprotonated. This deprotonation leads to an increase in the mean polarity of the polymer, which causes a shift in T_t to higher temperatures and a decrease in ΔH , since this parameter depends primarily on the amount of hydrophobic hydration around the polymer and this hydration mode is not possible in the vicinity of the charged carboxylate [5].

The bioconjugated Azo-NH₂ group decreases the transition temperature of the original polymer from 27.4 °C to 22.3 °C for polymer ELRGlu15 with a 59% conversion ratio (nine carboxyl groups have disappeared) due to the change in the global hydrophobicity of the polymer caused by this aromatic group [27]. More hydrophobic polymers lead to higher ΔH values as more water molecules are involved in hydrophobic hydration, thus lowering the transition temperature.

The influence of coupling ratio on T_t and ΔH has been studied with the *p*-phenylazobenzene derivatives obtained (azo-Glu15) under controlled conditions of pH. The resulting DSC curves show how, at pH 3, the transition temperature diminished linearly with the degree of coupling reaction, as expected from the decrease in the mean polarity of the polymer as the number of azo groups increases and the number of carboxyl groups decreases (see results shown in Fig. 4). Consequently, the transition temperature of the modified polymer could be correlated with its degree of substitution. Moreover, the temperature range in which this type of polymer can be used changes, and therefore so do its applications.

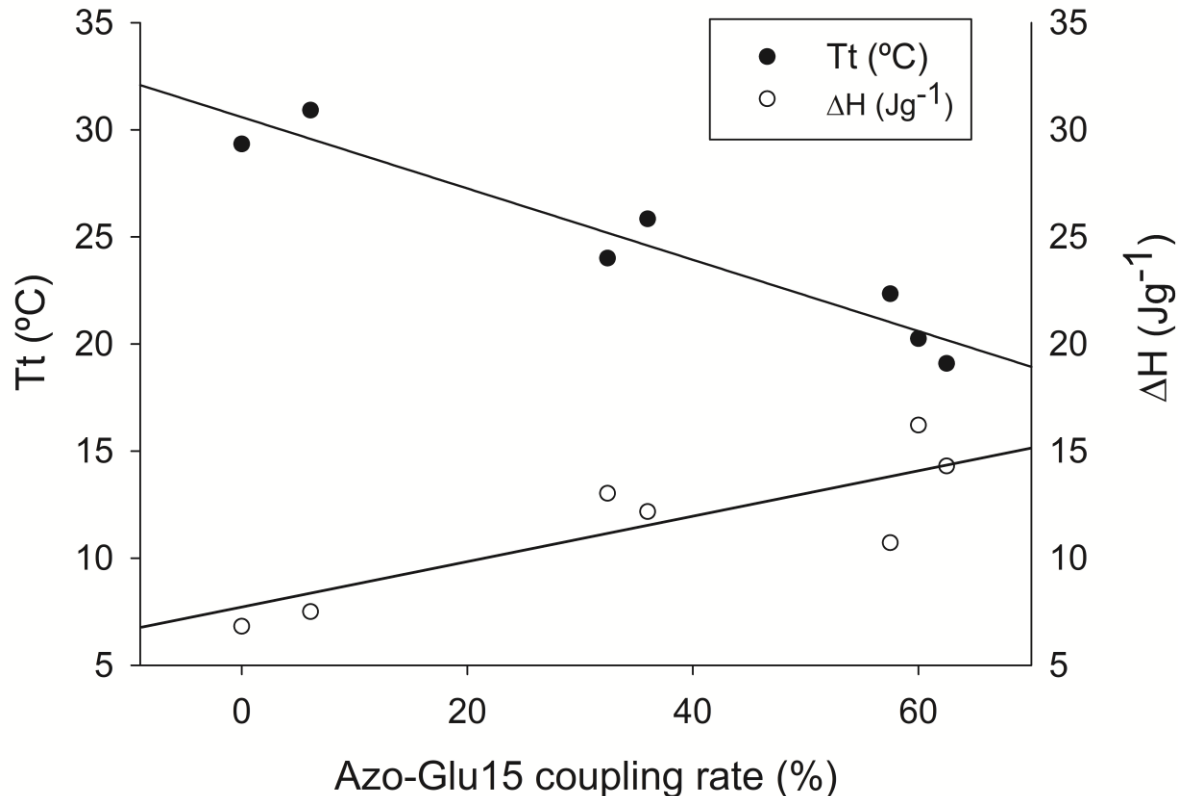


Figure 4. Transition temperatures (Tt) from DSC Analyses at pH 3 (5 °C/min after 10 min at 5 °C) for an aqueous solution of azo-Glu15 with different coupling rates (CR), obtained under different condition reactions (temperature, time or solvent) ($T_t = -0.1665 \text{ CR (\%)} + 30.596$; $R^2 = 0.9209$). Coupling ratio determined from absorbance measurements and MALDI-ToF spectral analysis.

The decrease in transition temperature observed for the ELRs is in agreement with the expected behavior for this family of macromolecules. Furthermore, the magnitude of this decrease is reasonable taking into account the Tt values reported in the literature for aromatic analogues of poly(VPGVG) with similar compositions [5]. Thus, the determination of Tt and ΔH for the differently modified Azo-Glu15 allows us to establish the relationship between Tt and the conversion ratio. This behaviour could be extrapolated to predict the Tt for other modified Azo-Glu15 with different conversion ratios. Moreover, the behaviour found in this study could be applied to other amine substrates in order to predict the conversion ratio needed to obtain modified ELRs with a suitable Tt for specific applications.

4. Conclusions

Our literature search revealed that very little previous work had been carried out in the area of enzyme-catalyzed post-polymerization functionalization, with most examples being hampered by low efficiency. Moreover, no esterification or amidation reactions have been carried out with proteins and using lipases as catalysts. In this study, a new method for the modification of ELRs has been developed with the aim of obtaining differently functionalized biopolymers that can exhibit further sensitivity in addition to their smart and

biocompatible nature, thus making them suitable for advanced applications. This new one-pot methodology consists of an amidation reaction between various functionalized amine reagents and an ELR bearing carboxylic groups along its chain. Chemical modification is carried out via enzymatic catalysis with *Candida antarctica* lipase B in a mild and effective one-pot reaction.

The coupling reaction with *p*-phenylazoaniline allowed us to obtain photoresponsive and electromagnetic radiation-sensitive biomaterials that could find a use as sensors in devices for controlled drug delivery, whereas coupling with 4-[(2-amino) carbamoyl]phenylboronic acid led a glucose-responsive ELR with potential for the design of glucose-sensitive biosensors and actuators for use in drug-delivery systems, for instance as insulin-delivery systems for patients with diabetes mellitus. A higher molecular weight molecule, namely PEG-NH₂, has also been enzymatically attached to the ELR, thus leading to the synthesis of a new type of branched ELRs.

In summary, we have reported an enzymatic method for the simple, fast and efficient modification of ELRs that could lead to the development of various types of stimuli-responsive, and hence “self-regulating”, systems (more commonly referred to as intelligent or smart materials) containing different functionalities bound via the free carboxylic groups present in the main chain.

5. Acknowledgements

The authors are grateful for financial support from the European Social Fund (ESF) and the European Regional Development Fund (ERDF), as well as funding from the EU (NMP-2014-646075, HEALTH-F4-2011-278557, PITN-GA-2012-317306 and MSCA-ITN-2014-642687), the MINECO (MAT2015-68901-R, MAT2016-79435-R and MAT2016-78903-R), the JCyL (projects VA244U13 and VA313U14), the CIBER-BBN, the JCyL, the Instituto de Salud Carlos III under the “Network Center of Regenerative Medicine and Cellular Therapy of Castilla and Leon”. The authors would like to thank Novozymes® for supplying a sample of CAL-B (Novozymes®-435) for use in this work and R. García for technical assistance in producing ELRGlu15.

6. References

1. S.A. Maskarinec, D.A. Tirrell, Protein engineering approaches to biomaterials design, *Curr. Op. Biotechnol.* 16 (2005) 422-426.
2. F.J. Arias, M. Santos, A. Fernandez-Colino, G. Pinedo, A. Girotti, Recent contributions of elastin-like recombinamers to biomedicine and nanotechnology. *Curr. Top. Med. Chem.* 14(6) (2014) 819-836. (JCR 3,402)
3. J.C. Rodríguez-Cabello, F.J. Arias, M. Alonso, A. Girotti, Elastin-like polypeptides in drug delivery, *Adv. Drug Delivery Rev.* 97 (2016) 85–100.
4. One-week glucose control via zero-order release kinetics from an injectable depot of glucagon-like peptide-1 fused to a thermosensitive biopolymer. *Nat. Biomed. Eng.* 1(6) (2017) 0078.
5. D.W. Urry, *What sustains life? A consilient mechanism for protein-based machines and materials*, first ed., Springer, New York, 2006.
6. R. Alvarez-Rodríguez, F.J. Arias, M. Santos, A.M. Testera, J.C. Rodríguez-Cabello, Gold tailored photosensitive elastin-like polymer: Synthesis of

- temperature, pH and UV-VIS sensitive probes, *Macromol. Rapid Commun.* 31 (2010) 568-573.
7. M. Rashidian, J. K. Dozier, M. D. Distefano, Enzymatic labeling of proteins: techniques and approaches. *Bioconjugate Chem.* 24 (8) (2013), 1277–1294.
 8. S. Sen, J.E. Puskas, Green Polymer Chemistry: Enzyme Catalysis for Polymer Functionalization, *Molecules* 20 (2015) 9358-9379.
 9. N. Karaki, A. Aljawish, C. Humeau, L. Muniglia, J. Jasniewski, Enzymatic modification of polysaccharides: Mechanisms, properties and potential applications. A review, *Enzyme Microb. Technol.* 90 (2016) 1-18
 10. M. Vastano, A. Pellis, B. Immirzi, G.D. Poggetto, M. Malinconico, G. Sannia, G.M. Guebitz, C. Pezzella, Enzymatic production of clickable and PEGylated recombinant polyhydroxyalkanoates, *Green Chem.* 19 (2017), 5494-5504.
 11. G. Brahmachari, Lipase-Catalyzed Organic Transformations: A Recent Update, in: G. Brahmachari, A. L. Demain and A. L. Jose (Eds.), *Biotechnology of Microbial Enzymes: Production, Biocatalysis and industrial applications*, first ed., Academic Press, 2017, pp 325-346.
 12. Z. Meng, W. Hou, H. Zhou, L. Zhou, H. Chen, C. Wu, Therapeutic Considerations and Conjugated Polymer- Based Photosensitizers for Photodynamic Therapy, *Macromol. Rapid Commun.* 39(5) (2018) 1700614.
 13. R.M. DiSanto, V. Subramanian, Z. Gu, Recent advances in nanotechnology for diabetes treatment, *WIREs Nanomed. Nanobiotechnol.* 7(4) (2015), 548-564.
 14. J. J. Kim, K. Park, Modulated insulin delivery from glucose-sensitive hydrogel dosage forms, *J. Control Release* 77 (2001) 39–47.
 15. R. Mehvar, Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation, *J. Pharm. Pharm. Sci.* 3 (2000) 125-136.
 16. A. Girotti, J. Reguera, F.J. Arias, M. Alonso, A.M. Testera, J.C. Rodríguez-Cabello, Influence of the Molecular Weight on the Inverse Temperature Transition of a Model Genetically Engineered Elastin-like pH-Responsive Polymer, *Macromolecules* 37 (2004) 3396-3400.
 17. A. Matsumoto, S. Ikeda, A. Harada, K. Kataoka, Glucose-responsive polymer bearing a novel phenylborate derivative as a glucose-sensing moiety operating at physiological pH conditions, *Biomacromolecules* 4(5) (2003) 1410-1416.
 18. E. Fletcher, D. Yan, A. Kosiba, Y. Zhou, H. Shi, Biotechnological applications of elastin-like polypeptides and the inverse transition cycle in the pharmaceutical industry, *Protein Expr. Purif.* 153 (2019) 114–120.
 19. E. Tejada-Montes, A. Klymov, M. Reza Nejadnik, M. Alonso, J.C. Rodríguez-Cabello, X.F. Walboomers, A. Mata, Mineralization and Bone regeneration using a bioactive elastin-like recombinamer membrane, *Biomaterials* 35 (2014) 8339-8347.
 20. M.J. Piña, A. Girotti, M. Santos, J.C. Rodríguez-Cabello, F.J. Arias, Biocompatible ELR based polyplexes coated with MUC1 specific aptamers and targeted for breast cancer gene therapy, *Mol. Pharm.* 13(3) (2016) 795–808.
 21. M. Alonso, V. Reboto, L. Guiscardo, A. San Martín, J.C. Rodríguez-Cabello, Spiropyran derivative of an elastin-like bioelastic polymer: Photoresponsive molecular machine to convert sunlight into mechanical work, *Macromolecules*, 33(26) (2000) 9480-9482.

22. V. Leonard, L. Fransson, S. Lamare, K. Hult, M. Graber, A water molecule in the stereospecificity pocket of *Candida Antarctica* lipase B enhances the enantioselectivity towards 2-pentanol, *Chem. Bio. Chem.* 8 (2007) 662–667.
23. H. Hirakawa, N. Kamiya, Y. Kawarayashi, T. Nagamune, Log P effect of organic solvents on a thermophilic alcohol dehydrogenase, *Biochim. Biophys. Acta* 1748 (2005) 94-99.
24. J. Iniesta, M.D. Esclapez-Vicente, J. Heptinstall, D.J. Walton, I.R. Peterson, V.A. Mikhailov, H.J. Cooper, Retention of enzyme activity with boron-doped diamond electrode in the electro-oxidative nitration of lysozyme, *Enzyme Microb. Technol.* (2010) 472-478.
25. J. Uppenberg, N. Ohrner, M. Norin, K. Hult, G.J. Kleywegt, S. Patkar, V. Waagen, T. Anthonsen, A. Jones, Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols, *Biochemistry* 34 (1995) 16838–16851.
26. K. Pavel, H. Ritter, *Enzymes in polymer chemistry*. 6. Lipase-catalyzed acylation of comb-like methacrylic polymers containing OH groups in the side chains, *Makromol. Chem. Macromol Chem. Phys.* 193 (1992) 323-328.
27. D.W. Urry, *Molecular machines. How motion and other functions of living organisms can result from reversible chemical changes*, *Angew. Chem. Int., Ed. Engl.* 32(6) (1993) 819-841.

SUPPORTING INFORMATION

Physicochemical characterization of the ELR and m-ELRGlu15

The **chemical structures** of functionalized polymer samples were confirmed by ^1H NMR, ^{13}C NMR and FT-IR spectroscopy and MALDI-TOF mass spectrometry

Although the peaks in the ^1H NMR spectra of the polymers were broad and not very informative, integration of the protons in the aromatic and aliphatic regions allowed us to confirm the respective coupling reaction.

The multiplet at 2.25-2.15 ppm (g) assigned to the methylene protons adjacent to the γ -carboxylic group of glutamic acid was less intense for m-ELR than for the original ELR as the $-\text{CH}_2-$ proton resonances shifted downfield from 2.34-2.14 to 2.14-1.61 ppm, as expected. The integration ratios of the aromatic and NH protons (m, 8.55-7.43) increased for azo-NH₂ and FB-NH₂ substrates, and a new band (m, 3.51-3.45) corresponding to the methylene protons, along with other new resonances at the expected positions, appeared for the PEG-NH₂ substrate, thus confirming ELR functionalization and the structure of m-ELRGlu15.

The modification ratio for Azo-Glu15 and FB-Glu15 was readily calculated from the ^1H NMR spectra by comparing the integration ratios of the N-H region for the initial ELR with that found for m-ELR, which includes the aromatic C-H's from the azobenzene or phenylboronic groups. Finally, the PEG-Glu15 coupling ratio was calculated from the ratio of the integration areas for the backbone methylene hydrogens in the PEG substrate [3.48 (m)] with the main chain methyl protons ($\text{CH}(\text{CH}_3)_2$) of valine residues. The

conversions obtained were similar to those obtained from absorbance measurements or from the corresponding MALDI-ToF.

The ^{13}C NMR spectra of the m-ELRGlu15s also supporting a structure in which Azo-NH₂, Sp-OH or FB-NH₂ aryl groups, or PEG-NH₂ moieties, are attached to the γ -carboxylic group of the glutamic acid residues found in the original biopolymer.

The carbonyl carbon of the glutamic residues (171.84 ppm) was replaced by the corresponding carbonyl from the amide/ester bond in m-ELRGlu15 (171.7, 171.54, 171.58 and 171.58 ppm) and significant shifts were detected for the α and β methylene groups in the glutamic residues of the starting material (from 56.2 and 27.76 ppm to 56.47 and 28.04 ppm. New resonances also appeared at the expected positions as a consequence of the enzymatic reaction, thus confirming the structure of the product.

The FTIR spectral analysis showed that, after modification with azo-NH₂, FB-NH₂ and PEG-NH₂, the m-ELRGlu15 presents an amide I band with a maximum close to 1625 cm^{-1} . The displacement of this peak (amide I) to lower frequency (azo, PEG and FB) indicates that the carboxylic groups in the ELR have reacted with the substrate. New peaks also appeared at the expected positions (1164, 976 or 838 cm^{-1} for azo-Glu15; 1342 and 975 cm^{-1} for FB-Glu15; and 1164 and 976 cm^{-1} for PEG-Glu15) as a consequence of the enzymatic reaction, thus confirming the structure of the product.

ELRGlu15

^1H NMR (400 MHz, DMSO-d₆): δ 8.17 (m, 152H, -NH-Gly(1,3)), 7.91 (m, 68H, -NH-Val(4)), 7.77 (m, 15H, -NH- Glu), 7.58 (m, 68H, -NH- Val(2)), 4.38-4.16 (m, 158H, CH-Val (4)+ α -CH- Pro + α CH-Glu), 4.16-4.04 (m, 68H, CH-Val(2)), 3.73 (m, 304H, -CH₂-Gly (1,3)), 3.55(m, 150H, δ CH₂-Pro), 2.25-2.15 (m, 30H γ CH₂-Glu), 2.08-1.66 (m, 466H, γ CH₂-Pro, CH(CH₃)₂-Val(2,4), - β CH₂-proline and β CH₂-Glu), 0.91-0.72 (m, 816H, CH(CH₃)₂).

^{13}C NMR (101 MHz, DMSO-d₆): δ 174.60, 172.32, 171,84, 171.69, 171.48, 170,41, 170.36, 169,26, 169.12, 59.96, 58.34, 56.10, 52.40, 47.69, 42.49, 41.98, 30.93, 30.61, 29.71, 27.76, 24.89, 19.60, 19.42, 19,37, 18.98, 18.90, 18.83, 18.66, 18.33, 18.13.

FT-IR (v/cm^{-1}): 3293, 3069, 2966, 2877, 1625, 1520, 1445, 1392, 1372, 1334, 1231, 1028, 929, 873, 667.

MALDI-Tof: 31943 Da

Azo-Glu15

^1H NMR (400 MHz, DMSO-d₆): δ 8.48-7.43 (m, 339,-NH and CH-arom), 6.86-6.74 (4H, CONH-azo), 4.43-4.06 (m, 238H, CH-Val(2,4),)+ α -CH- Pro + α CH-Glu + α CH-Glu-azo), 3.88-3.51 (m, 430H, -CH₂-Gly(1,3) and δ CH₂-Pro), 2.33-2.17 (m, 15H γ CH₂-Glu), 2.17-1.62 (m, 498H, γ CH₂-Pro, CH(CH₃)₂-Val(2,4), - β CH₂-proline, β CH₂-Glu and γ Glu-azo and β CH₂-Glu-azo), 0.95-0.71 (m, 822H, CH(CH₃)₂).

^{13}C NMR (101 MHz, DMSO-d₆): δ 177.91, 173.21, 172.70, 172.33, 171.48, 170.36, 169.12, 169.00, 152.45, 151.53, 147.81, 129.85, 129.44, 127.40, 124.10, 122.74, 119.63,

59.97, 58.35, 56.47, 56.10, (52,4), 47.69, 42.51, 41.99, 30.93, 30.61, 29.70, 25.80, 24.89, 24.70, 19.59, 19.43, 19.27, 18.99, 18.89, 18.81, 18.78, 18.76, 18.73, 18.66, 18.32, 18.14.

FT-IR (v/cm⁻¹): 3289, 3070, 2966, 2878, 1625, 1524, 1446, 1391, 1372, 1334, 1234, 1164, 1029, 976, 929, 838, 665, 547, 511.

FB-Glu15

¹H NMR (400 MHz, DMSO-d₆): δ 8.55-7.51 (m, 321H, -NH and CH-arom(FB)), 4.45-4.05 (m, 242H, CH-Val(2,4), α-CH- Pro + αCH-Glu/Glu-FB), 4.01-3.52 (m, 483H, -CH₂-Gly(1,3) + δCH₂-Pro+CONH-CH₂-CH₂(FB)), 2.40-2.23 (m, 19H B(OH)₂) 2.23-2.11(m,19H γCH₂-Glu), 2.12-1.62 (m, 556H, γCH₂-Pro, CH(CH₃)₂-Val(2,4), -βCH₂-proline and βCH₂-Glu/β, γ CH₂Glu-FB), 0.97-0.71 (m, 822H, CH(CH₃)₂).

¹³C NMR (101 MHz, DMSO-d₆): δ 172.32, 172.23, 172.04, 171.84, 171.77, 171.58, 171.48, 170.50, 170.35, 170.22, 169.65, 169.47, 169.12, 168.99, 168.91, 168.83, 168.59, 153.70, 109.99, 107.93, 59.96, 58.34, 56.47, 56.24, 56.10, 55.93, 53.22, 52.67, 49.99, 47.68, 42.50, 42.00, 32.05, 30.92, 30.61, 30.48, 29.70, 28.05, 25.86, 25.55, 24.89, 19.69, 19.59, 19.43, 19.35, 19.02, 18.99, 18.88, 18.83, 18.74, 18.69, 18.65, 18.31, 18.12.

FT-IR (v/cm⁻¹): 3291, 3070, 2965, 2936, 1626, 1523, 1446, 1392, 1335, 1233, 1028, 975, 929, 835, 667, 540, 523, 507.

PEG-Glu15

¹H NMR (400 MHz, DMSO-d₆): δ 8.43-7.57 (m, 306H, -NH), 4.37-4.07 (m, 235H, CH-Val (2,4)+ α-CH- Pro + αCH-Glu+CONH-CH₂-PEG), 3.89-3.53 (m, 421H, -CH₂-Gly (1,3)+ δCH₂-Pro), 3.51-3.45 (m, 62H, CH₂CH₂O), 3.23-3.20 (s, 5H, OCH₃), 2.14-1.64 (m, 485H, β,γCH₂-Glu, γCH₂-Pro, CH(CH₃)₂-Val(2,4), -βCH₂-proline and βγCH₂-Glu-PEG), 0.98-0.67 (m, 822H, CH(CH₃)₂).

¹³C NMR (101 MHz, DMSO-d₆): δ 172.32, 171.91, 171.47, 170.36, 170.28, 169.31, 169.11, 168.99, 168.91, 166.76, 70.22, 59.97, 58.34, 56.47, 56.11, 49.86, 47.69, 42.51, 41.99, 30.93, 30.61, 29.70, 27.51, 27.45, 24.89, 19.59, 19.47, 19.43, 19.33, 19.28, 18.99, 18.94, 18.89, 18.83, 18.65, 18.32, 18.12.

FT-IR (v/cm⁻¹): 3291, 3072, 2966, 2878, 1625, 1524, 1446, 1392, 1372, 1334, 1233, 1164, 1029, 976, 930, 837, 666, 545.

The purity and molecular weight of the polymers were determined from the corresponding MALDI-TOF and SDS-PAGE studies. The results confirmed structural changes in the original ELR as well as the coupling reaction ratio for the different polymers.

SDS-PAGE studies for ELR and m-ELRGlu15 allowed the identification of a major band around 32 kDa, which agrees with the expected molecular weight of the enzymatically

modified biopolymer. The intense band observed in SDS-PAGE at around the same value indicated by MALDI-TOF also demonstrates that the purification of m-ELRGlu15 was effective.

To further assess the purity and molecular weight of m-ELRGlu15, matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry was performed using a Voyager STR (Applied Biosystems) in linear mode and with external calibration using bovine serum albumin (BSA).

This technique confirmed the expected modified structure, as shown in Fig. 6, and allowed the coupling ratio to be determined from the weight average (Mw) (34,000-32,000) molecular masses of the products obtained by enzymatic modification.

We collect on Fig. 5 and 6, experimental results of m-ELRGlu15 with highest coupling ratio for the corresponding nucleophile among that obtained through the different assays carried out (data not shown). Best coupling ratio for PEG and FB was obtained with the same condition that has been optimized for phenylazobenzene. Coupling ratio for PEG-Glu15, Azo-Glu15 and FB-Glu15 are 12,8%, 59,5% and 60,8% respectively

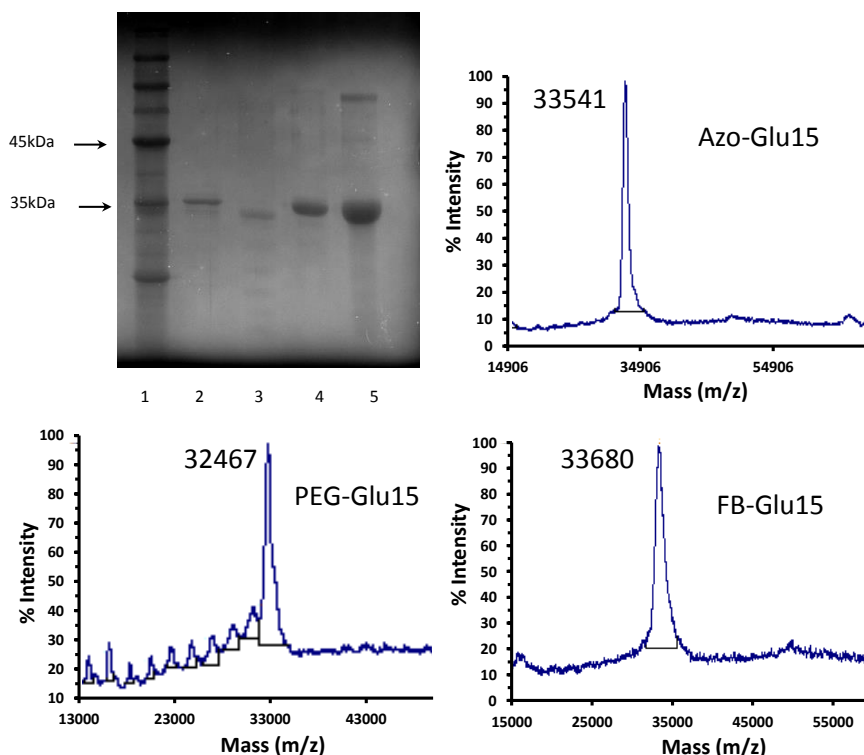


Figure 5. SDS-PAGE and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) for mELR. Line 1: Marker; line 2: PEG-Glu15; line 3: Azo-Glu15; line 4: ELR-Glu15; line 5: FB-Glu15

The coupling ratios for m-ELRGlu15s calculated from the MALDI-ToF spectra were very similar to the values calculated by ^1H NMR analysis and/or from absorbance measurements.

The thermal transitions for ELRGlu15 and m-ELRGlu15s were determined by **differential scanning calorimetry (DSC) analysis** using a Mettler Toledo 822^e DSC

instrument, as described in the Materials and Methods section. The DSC curves for aqueous solutions of these polymers at 50 mg mL⁻¹ prepared at pH 2 are shown in Fig. 6. It can be seen from this figure that the DSC endotherms for all m-ELRGlu15s prepared are similar, thus confirming that the modified biopolymers conserve their smart nature.

The conjugated Azo-Glu15 and FB-Glu15 groups decrease the transition temperature of the original polymer from 27.4 °C to 22.3 °C and 10.7 °C, respectively, due to the effect of these aromatic groups on the global hydrophobicity of the polymer [5]. More hydrophobic polymers have higher ΔH values due to the increase in the number of water molecules dedicated to hydrophobic hydration, which lowers the transition temperature.

DSC analysis of PEG-Glu15 showed that the transition temperature is higher than for the original biopolymer due to a decrease in global hydrophobicity caused by the PEG unit.

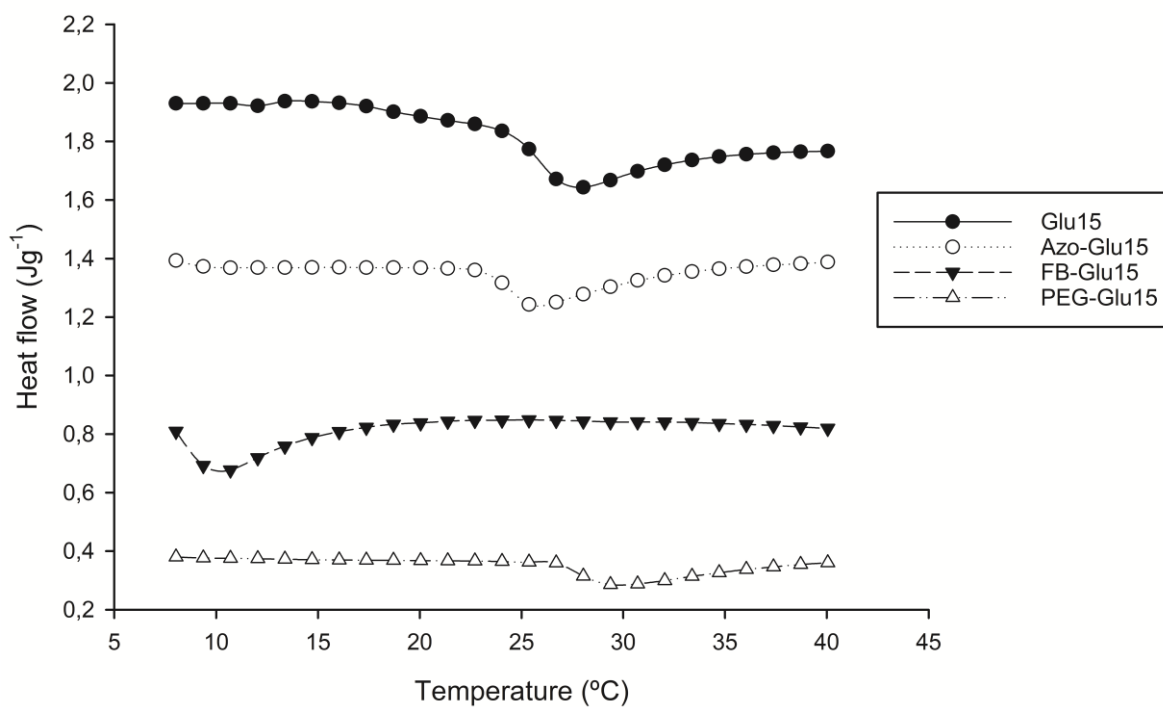


Figure 6. DSC endotherms (5 °C/min after 10 min at 5 °C) for aqueous solutions of the elastin-like recombinamer (ELRGlu15) and the enzymatically modified elastin-like recombinamer (m-ELR) at pH 2 and 50 mg·ml⁻¹ during the first heating scan.

CAPTIONS TO ILLUSTRATIONS

Figure 1. Proposed enzymatic modifications of ELRGlu15 at the free carboxylic group with different amines substrates using CAL-B as catalyst.

Figure 2. Time course of the enzymatic amidation of ELRGlu15 with azo-NH₂: (A) Influence of NH₂:γ-COOH ratio and (B) influence of enzyme quantity on amidation coupling ratio. Reactions were performed at 40 °C with a 1:1, 5:1 or 10:1 mol/mol ratio of amino groups to glutamic residues (A) or with different lipase concentrations, expressed as mg of catalyst per 100 mg of biopolymer (w/w %) (B). The solvent mixture is 2:1 DMF:THF. A control experiment in which no lipase was present was carried out, with no conversion being detected. All experiments were performed in triplicate.

Figure 3. Coupling of phenylazobenzene to the ELR as a function of the temperature and solvent used in the enzymatic reaction after reaction for 4 days. The error bars represent the standard deviation for triplicates. Reaction time: 4 days; ELR:Novozym 435 10:1 (w/w); ratio of amidation agent to carboxyl acid units: 5:1 mol/mol.

Figure 4. Transition temperatures (T_t) from DSC Analyses at pH 3 (5 °C/min after 10 min at 5 °C) for an aqueous solution of azo-Glu15 with different coupling rates (CR), obtained under different condition reactions (temperature, time or solvent) ($T_t = -0.1665 \text{ CR (\%)} + 30.596$; $R^2 = 0.9209$). Coupling ratio determined from absorbance measurements and MALDI-ToF spectral analysis.

Figure 5. SDS-PAGE and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) for mELR. Line 1: Marker; line 2: PEG-Glu15; line 3: Azo-Glu15; line 4: ELR-Glu15; line 5: FB-Glu15

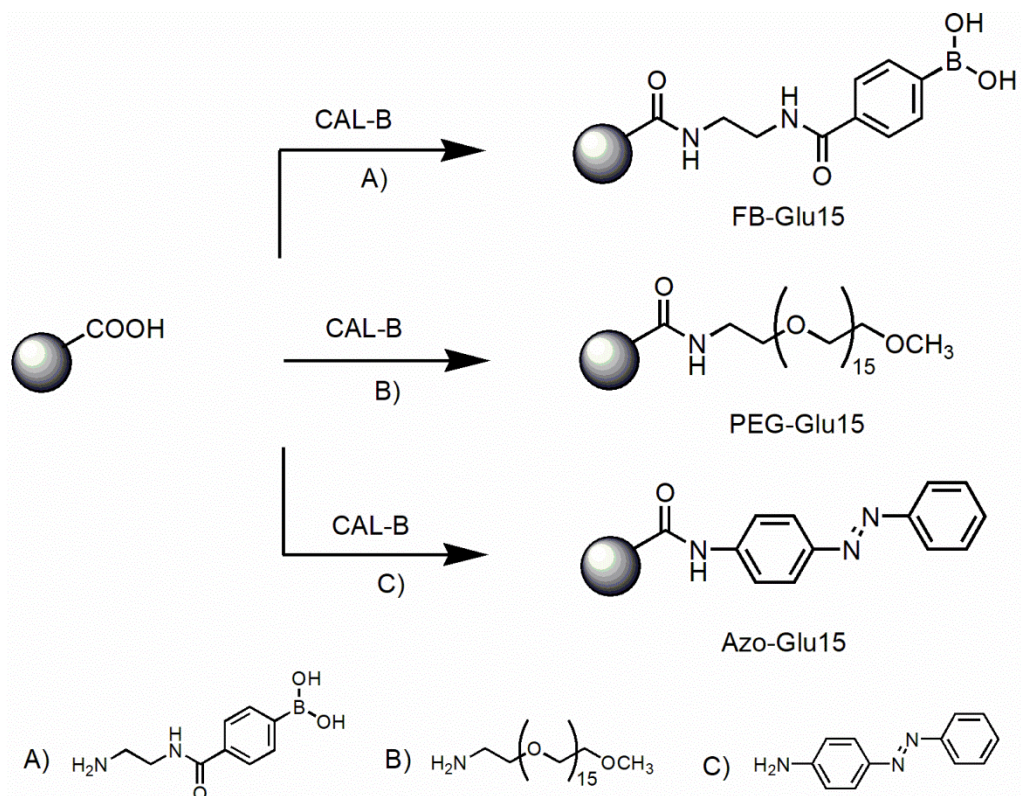
Figure 6. DSC endotherms (5 °C/min after 10 min at 5 °C) for aqueous solutions of the elastin-like recombinamer (ELRGlu15) and the enzymatically modified elastin-like recombinamer (m-ELR) at pH 2 and 50 mg•ml⁻¹ during the first heating scan

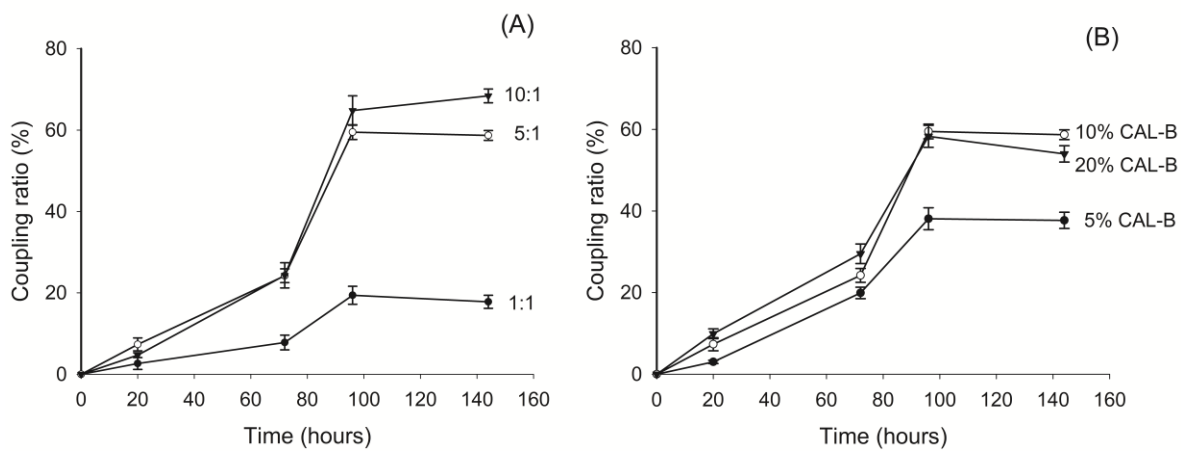
ILLUSTRATIONS

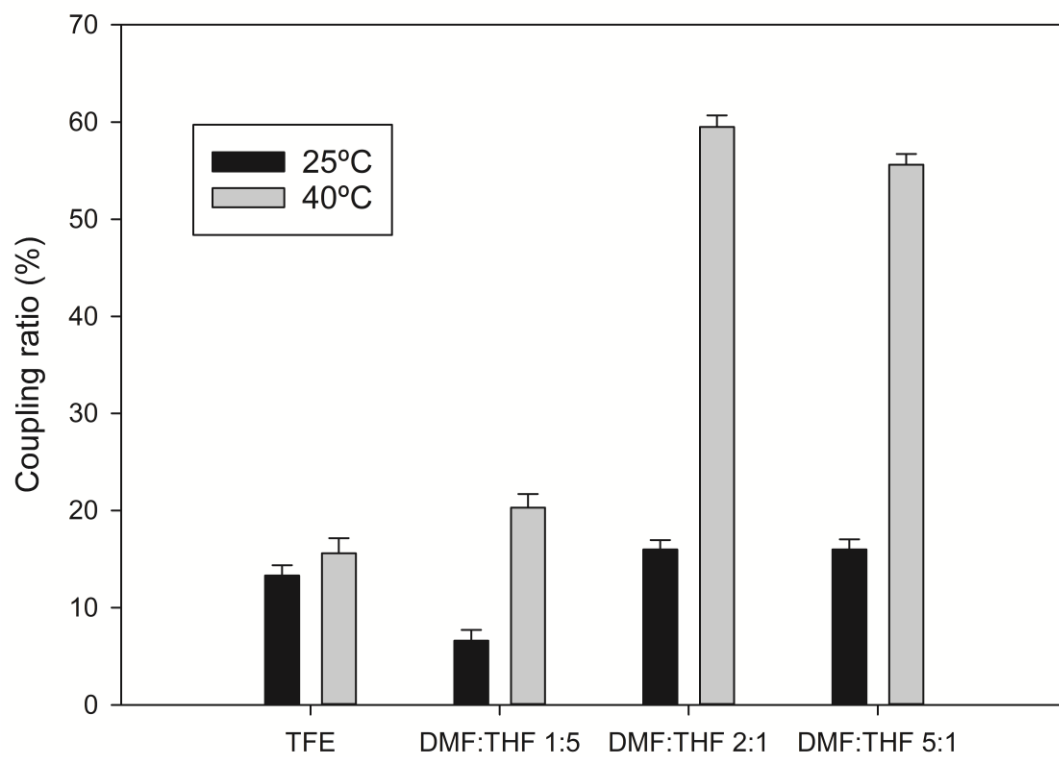
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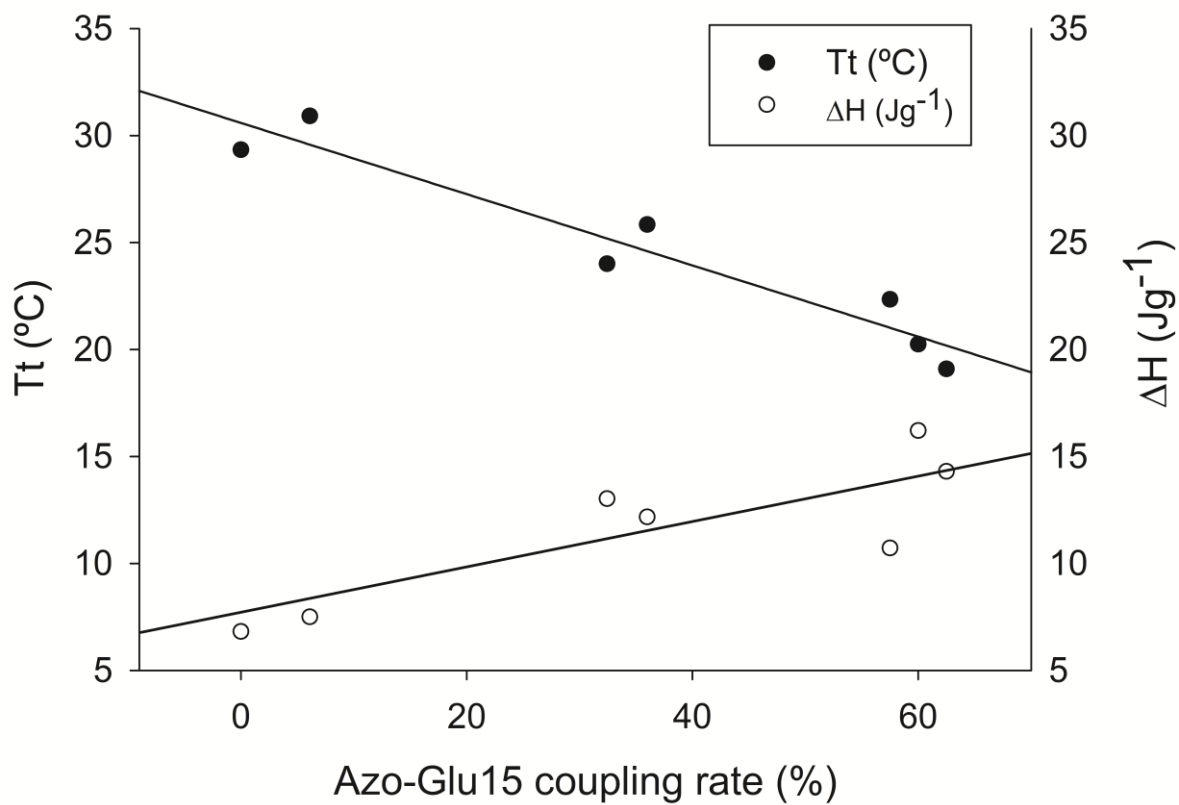
Ana M. Testera

Figure 1.









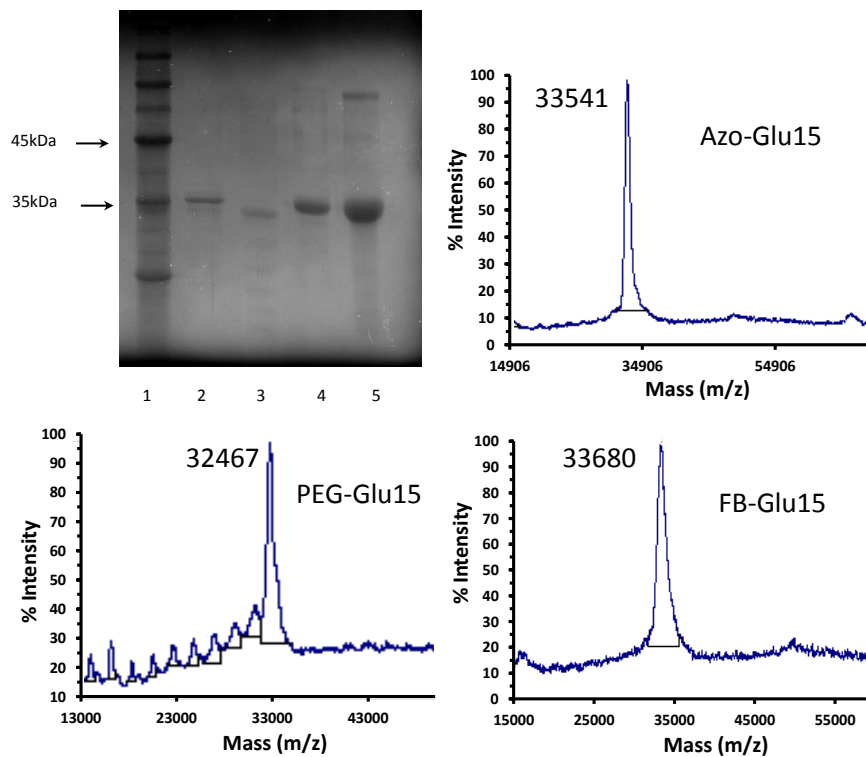


Figure 6.

