Design and bioproduction of a recombinant multi(bio)functional elastin-like protein polymer containing cell adhesion sequences for tissue engineering purposes

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Genetic engineering techniques were used to design and biosynthesise an extracellular matrix (ECM) analogue. This was designed with a well-defined molecular architecture comprising different functional domains. The structural base is a elastin-derived repeating unit, which confers an adequate elastic characteristic. Some of these elastin domains have been modified to contain lysine; this amino acid can be used for crosslinking purposes. The polymer also contain periodically spaced fibronectin CS5 domains enclosing the well-known cell attachment sequence REDV. Finally, the polymer has target sequences for proteolitic action. These sequences are those found in the natural elastin and are introduced to help in the bioabsorption of the polymer. In addition, these proteolitic sequences were chosen in a way that, after proteolitic action, the released fragments will be bioactive. These fragments are expected to promote cell proliferation activity, angiogenesis and other bioactivities of interest for tissue growing, repairing and healing.

After purification, the resulting polymers proved to be of high purity and correct sequence. Glutaraldehyde has shown to be a cross-linking agent for this polymer, yielding insoluble hydrogel matrices. This work is framed in a long term project aimed to exploit the power of genetic engineering for the design and bioproduction of complex ECM analogues showing the rich complexity and multi (bio)functionality of the natural matrix.

Introduction

One of the main tasks in the development of tissue engineering is the advance in the design and production of materials designed to act as adequate scaffold for the growing cells and tissues. The evolution in the development of artificial extracellular matrices begun with the use of biotolerated and biodegradable synthetic materials, mostly polymers, which showed cell attachment and spreading capabilities of a rather unspecific nature [1]. Soon, these materials were improved. Some macromolecules of natural origin were used but more important, more specific functionalities were included in their structure, specially peptide cell attachment sequences [1]. The use of these sequences significantly increased the performance of those matrices. However, in spite of the improvement in cell adhesion, these

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synthetic scaffolds are still far from showing the efficiency and rich complexity and functionality of the natural ECM.

More recently, the development of genetic engineering has allowed the design and bioproduction of protein polymers. These are mainly made from repeating sequences found in natural proteins, such as elastin, silks, etc., and selected modifications [2, 3]. One of the most promising family of protein polymers is the elastin-like family. The most renowned building block within this family is the pentapeptide (VPGZG), where Z can be any natural or modified amino acid [4]. The enormous potential of this family for biomedical uses, and in particular in tissue engineering, has been demonstrated by Urry and co-workers in a pioneer effort [3]. Elastin-like polymers (ELPs) have shown an outstanding biocompatibility. Apparently, the immune response systems of the body cannot differentiate the ELPs from the endogenous elastin [5]. In addition, due to their protein nature, their bioabsorption proceeds by conventional metabolic routes, yielding just natural amino acids.

The matrices resulting from cross-linking of ELPs show a mechanical response quite similar to the natural elastin [6]. This factor is of importance for tissue engineering. When properly attached to the ECM, cells sense the forces to which they are subjected via integrins and respond by producing an ECM that will withstand those forces. Therefore, the artificial ECM has to properly transmit forces from the environment to the growing tissue. Only in this way, the new tissue will build the adequate natural ECM that eventually will replace the artificial ECM.

In addition, the ELPs have an acute ''smart'' nature. Below a given temperature, the transition temperature (T_t), the uncrosslinked polymer chains are soluble in water. However above T_t the polymers starts a complex self-assembling process that leads to an aggregation of the polymer chains, initially forming nano- and microparticles, which segregates from the solution [4]. Although, this smart nature is not of particular relevance in the final use of ELPs as ECM, it enormously simplifies the purification procedure of the bioproduced ELPs and some of the intermediate steps in the preparation of the ECM.

ELP containing cell attachment sequences (RGD and REDV) were first chemically synthesised by Urry and coworkers [7, 8]. More recently, to overcome the huge effort needed to produce this kind of ELPs by conventional chemical methods, the development of a genetic engineering approach has proven to be a powerful tool for the design and production of complex protein polymers with well-defined and controlled sequence. However, the potential of this technique is still far from being fully exploited in the development of sophisticated ECM for tissue engineering in a relatively simple and inexpensive manner. Up to now, the bioproduced ELPs are not much more than analogs of these first examples [9–12]. However, the natural ECM is known to contain a rich variety of functions which are not restricted to confer adequate mechanical response while having a certain type of cell attachment sequences. This works was intended as being one step more towards the development of a complex and multifunctional artificial ECM that mimics the versatility and complexity of the natural ECM. The design, bioproduction and physical–chemical characterisation of a polymer designed with this idea is presented in this work. Further biological tests will appear elsewhere. This effort represents the initial stage of a more ambitious and long-termed project aiming to the development of complex ECM tailored for specific or more general tissue engineering purposes, showing multifunctionality and multibioactivities.

Experimental

Materials

Escherichia coli strain BLR(DE3) and pET-25(+) were obtained from Novagen (Madison, WI). *Taq* DNA polymerase and *Eam* 1104 I restriction enzyme were purchased from Stratagene (La Jolla, CA). Ampicillin and isopropyl-1-β-D-thiogalactoside (IPTG) were obtained from Apollo Scientific (Bredbury, UK) and Tetracycline was obtained from Roche Diagnostics (Basel, Switzerland). Synthetic oligonucleotides were purchased from IBA GmbH (Goettingen, Germany). PCR amplifications were carried out in a Perkin-Elmer GeneAmp 2400 thermal cycler. Gel images were captured by a Kodak DC-120 digital camera.

Synthetic gene construction

Cloning and molecular biology techniques were performed using standard procedures and the sequence of all putative inserts was verified by automated DNA sequencing. A synthetic DNA duplex encoding the peptide monomer depicted in Fig. 1(a) and flanked by inverted *Eam* 1104 I recognition sites was generated by polymerase chain reaction (PCR) amplification using synthetic oligonucleotides (see Fig. 1(b)). The gene sequence was selected to emphasise *E. coli* preferred codons while also minimising sequence repetition. After gene cloning, the monomer was generated by digestion with *Eam* 1104 I, isolated and subjected to concatenation ligation reaction (see Fig. 2). The concatenamer mixture was cloned and the transformants were selected by PCR colony screening. Several plasmids were selected based on their insert length (see Fig. 3) and the corresponding concatamers were subcloned into a modified pET-25(+) expression vector. The recombinant plasmids were transformed into the expression strain BLR(DE3).

Expression and purification

Bacterial cultures were grown at 37 °C in LB medium containing 100 μg/ml ampicillin and 30 μg/ml tetracycline. Gene expression was induced by the addition of IPTG to 1 mM at an OD_{600} of 0.8. The cultures were incubated an additional 3 h, harvested by centrifugation, resuspended and lysed by ultrasonic disruption. Insoluble debris was removed by centrifugation and the cleared lysate was subjected to several cycles of cold (4 °C) and warm (50 °C) centrifugation [16]. All the purification steps were carried out in 0.10 M sodium phosphate buffer (pH = 7.02).

A

(VPGIG)$_2$VPGKG(VPGIG)$_2$**EEIQIGHIPREDVDYHLYP**(VPGIG)$_2$VPGKG(VPGIG)$_2$(VGVAPG)$_3$

B

```
  1                V  P  G    I  G  V    P  G  I  G    V  P  G
  1 ACCACTCTTC AGTACCGGGC ATCGGTGTTC CGGGCATTGG TGTGCCGGGC
    TGGTGAGAAG TCATGGCCCG TAGCCACAAG GCCCGTAACC ACACGGCCCG

 14  K  G  V    P  G  I  G    V  P  G    I  G  E    E  I  Q  I
 51 AAAGGTGTTC CGGGCATTGG TGTGCCGGGC ATTGGTGAAG AAATCCAGAT
    TTTCCACAAG GCCCGTAACC ACACGGCCCG TAACCACTTC TTTAGGTCTA

 31    G  H  I    P  R  E    D  V  D  Y    H  L  Y    P  V  P
101 CGGGCATATC CCACGCGAGG ATGTGGACTA CCACCTGTAT CCGGTGCCAG
    GCCCGTATAG GGTGCGCTCC TACACCTGAT GGTGGACATA GGCCACGGTC

 47 G  I  G  V    P  G  I    G  V  P    G  K  G  V    P  G  I
151 GCATTGGTGT GCCGGGCATT GGTGTTCCGG GCAAAGGTGT GCCGGGCATT
    CGTAACCACA CGGCCCGTAA CCACAAGGCC CGTTTCCACA CGGCCCGTAA

 64  G  V  P    G  I  G  V    G  V  A    P  G  V    G  V  A  P
201 GGTGTTCCGG GCATCGGTGT GGGCGTTGCG CCGGGTGTGG GCGTTGCCCC
    CCACAAGGCC CGTAGCCACA CCCGCAACGC GGCCCACACC CGCAACGGGG

 81    G  V  G    V  A  P    G  V
251 GGGTGTGGGC GTTGCACCGG GTGTAAGAAG AGTGGT
    CCCACACCCG CAACGTGGCC CACATTCTTC TCACCA
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Figure 1 (A) Amino acid sequence of the monomer. The CS5 peptide sequence is highlighted in bold. Cell binding motif is underlined. (B) Nucleotide sequence and deduced amino acid sequence of the polymer monomer. Restriction sites flanking the translated region are highlighted in italics.

Turbidity

Turbidity experiments were conducted in a Varian Cary 50 UV-Vis spectrophotometer with a thermostatised sample chamber. Turbidity was assessed by the change in absorbance at 300 nm for a 50 mg/ml polymer solution in 0.10 M sodium phosphate buffer (pH = 7.02). At a given temperature, the sample was left until a constant turbidity value was reached. This steady value is considered as the actual turbidity for the sample at that temperature.

Differential scanning calorimetry (DSC)

DSC experiments were performed on a Mettler Toledo 822^e with liquid-nitrogen cooler. Calibration of both temperature and enthalpy was made with a standard sample of indium. For DSC analysis, 50 mg ml^{-1} phosphate buffered (0.1 M) water solutions (pH = 7.02). In a typical DSC run, 20 µl of the solution were placed inside a standard 40 µl aluminium pan hermetically sealed. The same volume of water was placed in the reference pan. All DSC samples were pretreated 15 min at 5 °C inside the sample chamber just before the beginning of the experiment.

Cross-linking

The cross-linked procedure used was the one described by Welsh and Tirrell [11]. Briefly, a polymer film was cast from an DMSO solution and dried in vacuo (50 °C, 2 h) onto a circular aluminium mould (25 mm diameter).

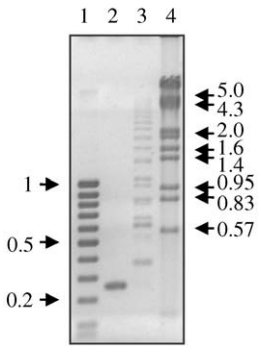

Figure 2 Agarose (1.5%) gel electrophoresis of the concatenation reaction of monomer gene. Lanes 1 and 4: Molecular standards. Lane 2: monomer gene. Lane 3: products of the concatenation reaction of the monomer gene. Arrows and numbers indicate the position and size of the molecular weight markers (in kbp).

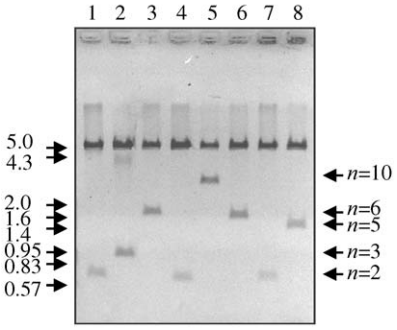

Figure 3 Agarose (1%) gel electrophoresis showing an example of the screening of plasmids containing the polymer genes. The plasmids were digested with two restriction endonuclease enzymes. The digestions produced a vector fragment (5547 bp) and a polymer gene fragment. On the left it is indicated the positions and sizes (in kbp) of the molecular weight markers. The number of repeating monomer genes of each polymer gene is labelled on the right.

The mould with the dried film was transferred to a closed chamber containing a pool of 12.5% glutaraldehyde solution (Sigma-Aldrich, Spain). Vapour phase cross-linking was carried out at room temperature for 18–24 h. The films were then treated with GTA solution (10 mM in PBS, pH = 7.4) for 2 h. Finally, the cross-linked films were treated with 0.2 wt % dimedone (Sigma-Aldrich, Spain) in PBS, pH = 7.4 for 24 h to quench any reactive residues in the matrix. The cross-linked matrices were thoughtfully washed with sterile PBS, pH 7.4 and stored at 4 °C in the same medium.

Results and discussion

The protein polymer studied in this work has been designed aiming to its use in the construction of bioactive matrices for tissue engineering. Four different types of building blocks has been integrated in its sequence in order to achieve an adequate balance of mechanical and bioactive responses. First, the final matrix is to be designed to show a mechanical response comparable to the natural extracellular matrix, so they are produced over a base of an elastin-like polymer of the type $(\mathrm{VPGIG})_n$. Along with the desired mechanical behaviour [12, 13], this base has shown an outstanding biocompatibility [5]. The second building block is a variation of the first. It has a lysine substituting the isoleucine so the lysine γ-amino group can be used for cross-linking purposes while retaining the properties of elastin-like polymers. The third group is the CS5 human fibronectin domain. This contains the well-known endothelial cell attachment sequence, REDV, immersed in its natural sequence to retain its efficiency [14]. In addition, the polymer also contains elastase target sequences to favour its bioprocesabilitty by natural routes. The chosen elastase target sequence is the hexapeptide VGVAPG, which is found in natural elastin. Its bioactivity is not restricted to being an inert target of protease activity. It is well known that these hexapeptides, as they are released by the protease action, have strong cell proliferation activity and other bioactivities related to tissue repairing and healing [15].

Once the design was decided, it is evident that conventional chemical synthesis is not adequate to face the production of this desired polymer. Therefore, the alternative route via the genetic engineering of bacterial strains, as described in the Experimental section, looked to be a better option for this purpose. The results of this method of bioproduction are described in the following paragraphs.

The bioproduced polymer is based on the monomer described in Fig. 1. The concatemerisation process and PCR colony screening (see Figs. 2 and 3) allowed the isolation of modified bacterial strains able to produce a particular *n*-mer with a monodisperse molecular weight. Several colonies, one of each able to produce a polymer with an particular polymerisation degree, were identified and selected. These colonies cover a polymerisation degree range from the monomer up to the decamer. Although all these colonies were preserved, only the characterisation of the highest molecular weight polymer (decamer) is presented in this work.

Expression of the polymer $(n = 10)$ can be easily differentiated from the expression of constitutive bacterial proteins. Examination of the whole cell lysates (see Fig. 4) reveals that the expression of the polymer takes place only before IPTG induction and achieving a maximum at about 3–4 h after induction.

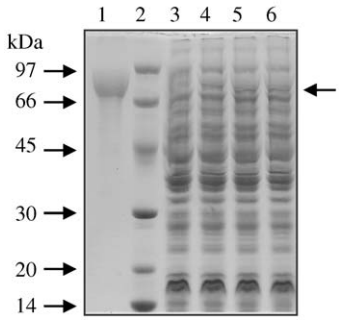

Figure 4 Analysis of polymer and bacterial extracts expressing the polymer by SDS-polyacrilamide gel electrophoresis in the presence of 2-mercaptoethanol and Coomassie brilliant blue staining. Lane 1: Purified polymer. Lane 2: Molecular standards. Lane 3: Bacterial extract containing the polymer gene before induction. Lane 4: Bacterial extract containing the polymer gene after 1 h induction. Lane 5: Bacterial extract containing the polymer gene after 3 h induction. Lane 6: Bacterial extract containing the polymer gene after 24 h induction. Numbers on the left indicate the corresponding apparent molecular weight values of the standard (in kDa). The arrow on the right indicates the position of the polymer.

One main characteristic of the ELPs is its ''smart'' nature. As mentioned in the introduction, ELPs are soluble in water below T_t and segregate from the solution above that temperature. This differential behaviour with temperature has been exploited in this work to purify the polymer from the bacterial lysate. The general method described by Urry [16] for recombinant ELPs purification has been used for this purpose. A purified sample of the polymer has been also included in the SDS–PAGE electrophoresis shown in Fig. 4. After purification, the bioproduction yield was about 100 mg/l. This value showed some dependence with the molecular weight (result not shown).

The verification of the correctness of the composition and molecular weight of this bioproduced polymer is carried out by amino acid analysis and mass spectroscopy. Fig. 5 shows a MALDI-TOF mass spectrum of the polymer $(n = 10)$. The experimental molecular weight found by this technique matches well the theoretical

TABLE I Theoretical and measured amino acid composition of the bioproduced polymer

Residue	Theoretical	Measured
Asp	2.28	2.38
Ser	0.10	0.05
Glu	3.53	4.62
Gly	30.81	29.34
Ala	3.42	5.12
Val	19.50	19.60
Met	0.10	0.23
Ile	12.55	12.94
Leu	1.35	0.58
Tyr	2.28	2.23
His	2.28	1.97
Lys	2.28	2.25
Arg	1.14	1.14
Pro	17.22	17.43

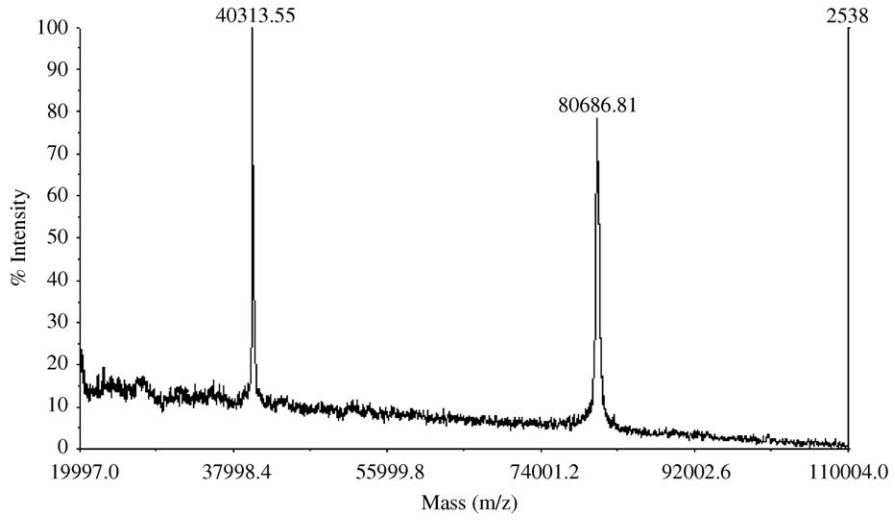

Figure 5 Mass spectra (MALDI-TOF/MS) of the polymer with $n = 10$. The expected mass of the polypeptide is 80695 Da. Signal at 40313 Da is assigned to doubly charged species.

molecular weight of the polymer (80695 Da) within the experimental error. Additionally, the measured amino acid content fits well with the theoretical composition (see Table I). In conclusion, all the characterisation tests point to the bioproduction of a polymer with the desired composition, sequence and molecular weight.

The smart nature of the polymer is also apparent and is an additional indirect evidence of the correctness of the molecular composition, since small modifications of the elastin-like sequence cause the lost of the smart behaviour [4]. Fig. 6 shows a temperature profile of aggregation of the polymer in 0.1 M PBS (pH = 7.02). This profile is based on turbidity measurements since the polymer solution remains clear below T_t, while, above T_t, the segregation of the polymer into a separate phase yields an enormous increase in the sample turbidity. The T_t can be identified as the temperature yielding a 50% turbidity. By this mean, the temperature profile shown in Fig. 6 indicates a $T_t = 20.1\,^{\circ}\mathrm{C}$. Taking in mind the purification protocol used form this polymer, this temperature is well within the particular temperatures (4 °C and 50 °C) used in the purification steps. Therefore, and according to Fig. 6, the polymer is completely dissolved at 4 °C and completely precipitated at 50 °C, as needed in the purification protocol.

Calorimetrically, this transition can be also characterised. Fig. 7 shows a DSC thermogram of a 50 mg/ml polymer solution in 0.1 M PBS (pH = 7.02). The thermogram shows the characteristic endotherm of the ELPs [4]. The peak temperature can be identified with T_t. This calorimetric T_t is similar to the one found in

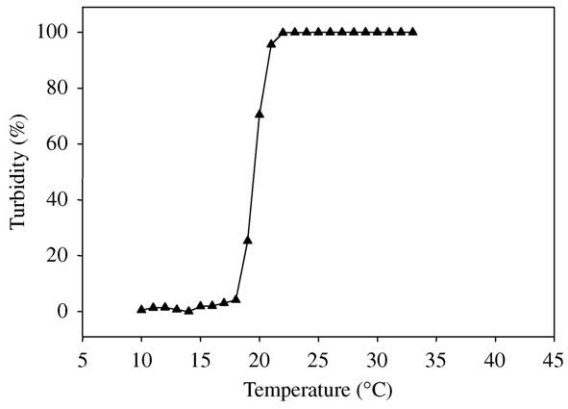

Figure 6 Turbidity profiles for a 50 mg/ml polymer solution in 0.1 M sodium phosphate buffer (pH = 7.02).

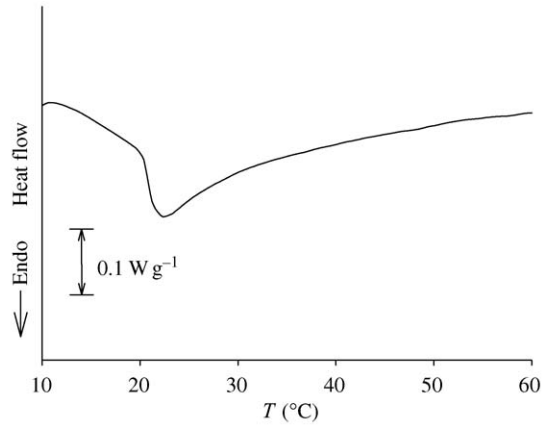

Figure 7 Typical DSC run of a 50 mg/ml polymer solution in 0.1 M sodium phosphate buffer (pH = 7.02). A heating rate of 10 °C/min was used in this example.

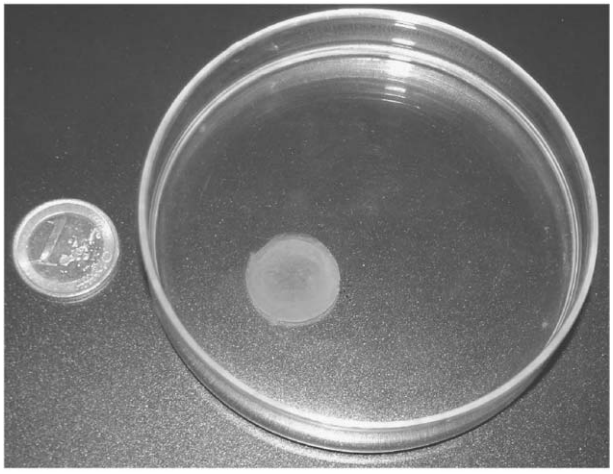

Figure 8 Photograph of a cross-linked matrix of the bioproduced polymer ($n = 10$). The GTA was used as cross-linker as described in the experimental section.

turbidity the turbidity profiles. The small differences can be attributed to the inherent thermal lags of the dynamic DSC measurement. The peak integral represents the characteristic transition enthalpy (ΔH_0) of this polymer. The value found from calorimetric data is $\Delta H_0 = 3.5$ J/g. Both calorimetric values, T_t and ΔH_0, are reasonable as compared to other ELPs, taking into account the net charge of the polymer and its mean polarity [4, 17].

Finally, to test the capacity of the polymer to be cross-linked into solid matrices we have used the well known GTA cross-linker. This has the advantage that can be used in vapour-phase cross-linking. Fig. 8 shows and example of a circular film (about 1 mm thick) obtained by this technique. The final cross-linked matrix is a swollen hydrogel with good mechanical characteristics, insolubility and consistency. In addition, the γ-amino residue of lysine is very versatile as cross-linking point. This same residue has been used for cross-linking purposes in polymers of similar chemical characteristics. Solution protocols use bis(sulfosuccinimidyl) suberate and disuccinimidyl suberate [12] as cross-linkers, and even enzymatic cross-linking by, for example, lysyl oxidase [9] can be used for this purpose.

Conclusions

Genetic engineering of protein polymers has proven to be a very adequate way to produce complex polymers with well defined sequence and control of the molecular architecture. This method open the possibility to obtain complex designs that mimics the rich complexity in functionality and bioactivity of the natural ECM. The use of elastin-like polymers as the structural base of the polymer helps in achieving adequate mechanical and biocompatibility properties. Additionally, their smart nature can be used to simplify the purification protocols and adds an interesting additional control tool in the intermediate steps of the matrix preparation or in designing advanced systems based in these polymers. Finally, the existence of the γ-amino residues of lysine are adequate to obtain cross-linked matrices with good properties for their use as scaffolds in tissue engineering.

Acknowledgment

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