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Enzymatic cross-linking of human recombinant elastin (HELP) as biomimetic approach in vascular tissue engineering

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Abstract The use of polymers naturally occurring in the extracellular matrix (ECM) is a promising strategy in regenerative medicine. If compared to natural ECM proteins, proteins obtained by recombinant DNA technology have intrinsic advantages including reproducible macromolecular composition, sequence and molecular mass, and overcoming the potential pathogens transmission related to polymers of animal origin. Among ECM-mimicking materials, the family of recombinant elastin-like polymers is proposed for drug delivery applications and for the repair of damaged elastic tissues. This work aims to evaluate the potentiality of a recombinant human elastin-like polypeptide (HELP) as a base material of cross-linked matrices for regenerative medicine. The cross-linking of HELP was accomplished by the insertion of cross-linking sites, glutamine and lysine, in the recombinant polymer and generating ε-(γ-glutamyl) lysine links through the enzyme transglutaminase. The cross-linking efficacy was estimated by infrared spectroscopy. Freeze-dried cross-linked matrices showed swelling ratios in deionized water ($\approx$2500%) with good structural stability up to 24 h. Mechanical compression tests, performed at 37°C in wet conditions, in a frequency sweep mode, indicated a storage modulus of 2/3 kPa, with no significant changes when increasing number of cycles or frequency. These results demonstrate the possibility to obtain mechanically resistant hydrogels via enzymatic crosslinking of HELP. Cytotoxicity tests of cross-linked HELP were performed with human umbilical vein endothelial cells, by use of transwell filter chambers for 1–7 days, or with its extracts in the opportune culture medium for 24 h. In both cases no cytotoxic effects were observed in comparison with the control cultures. On the whole, the results suggest the potentiality of this genetically engineered HELP for regenerative medicine applications, particularly for vascular tissue regeneration.

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1 Introduction

Synthetic proteins are emerging as a new class of biomaterials. With respect to the use of traditional natural polymers, the recombinant DNA technology approach results in a reproducible composition, sequence and molecular mass of the protein, overcoming the potential pathogens transmission related to natural polymers. Furthermore, genetic engineering allows a precise control at a molecular level so that the production of finely tuned and tailored artificial proteins can be achieved.

The availability of methods to exactly order peptide domains into the artificial protein, and then to control its properties, opens new application opportunities for the use of these polymers in bionanotechnology and regenerative medicine [1, 2].

The extracellular matrix (ECM) is a composite material which contains a complex mixture of fibrous proteins and

heteropolysaccharides, providing an important model for the design of biomaterials [3, 4]. The goal of mimicking the structure of ECM and its biological functions requires the design of artificial substrates that reproduce one or, if possible, more of the properties and functionalities of natural tissues. With the recombinant DNA technology it is now possible to synthesize proteins analogues of ECM ones [5], largely investigated and proposed for different biomedical applications [1].

Among ECM-mimicking materials, the family of recombinant elastin-like polymers [1] are inspired by the amino acidic sequence of native elastin, one of the most abundant ECM proteins, that plays an essential role in tissue biomechanics and modulates a variety of cellular response [6]. Due to these remarkable properties, elastin-like polymers have been proposed as appealing materials for tissue engineering and regenerative medicine [7–9].

A peculiar and extensively studied characteristic of these elastic proteins is coacervation (or "self assembly" property). Under appropriate conditions of concentration, ionic strength and increasing temperature, the protein is able to separate from the solution as a second phase. Urry and co-workers showed that the physical properties of repetitive elastin-like polypeptides (ELP) are highly dependent on the amino acidic composition of the peptide repeat [10, 11]. It has been shown that the self assembly behaviour is mainly due to the presence of the hydrophobic pentapeptide motif (VPGXG), typical of the mammalian protein [10, 11]. Physical or non covalently crosslinked networks, produced through the self-association of chemically similar domains [12], overcome the use or purification from harsh chemicals, often related to adverse cellular response. On the other end, these networks could be not stable over a wide range of pH and temperatures, and therefore unable to offer the mechanical integrity ensured by a chemically cross-linked system. Elastin-like based hydrogels are extensively proposed for biomedical/pharmaceutical applications such as controlled drug release and delivery, tissue engineering, and regenerative medicine [13, 14]. In particular, they could be useful for vascular tissue engineering because elastin, a major constituent of the medial layer of the arterial wall, is responsible for the elasticity and recoiling efficiency of the blood vessels. Although several attempts to improve elastin biosynthesis have been made, most engineered vessels lack mature elastin fibres or presents inadequate contents of the protein that is poorly organized [15–17].

To obtain chemically cross-linkable systems, and improve mainly the mechanical properties, ELP have been appropriately designed [18–20] to include precise sequences with available amino groups prone to react with different species. Soluble forms of elastin, including α-elastin, recombinant tropoelastin and engineered recombinant ELP have been chemically crosslinked, by using a number of chemical cross-linkers, including aldehydes [12, 21–24], quinones [25, 26], succinimidyl esters [6, 18, 24, 27–29], carbodiimides [30], phosphines [31, 32], isocyanates [33, 34], genipin [27, 35], and ethers [36]. Methods based on the use of enzymes [19], UV/visible light activated photoinitiators [37] and γ-irradiation [30, 38] have been investigated as alternative approaches to crosslink synthetic elastin polymers. Among these methods, enzymatic crosslinking allows to control the specificity of the reaction of the protein and occurs in conditions close to the natural ones. This can lead to in vivo cross-linking of the proteins [39]. Transglutaminase catalyzes an acyl-transfer reaction between the ε-amino group of a protein bound lysine residue and the γ-carboximide group of a protein bound glutamine residue, therefore a covalent cross-link, in the form of an isopeptide bond, is formed.

Up to now, the majority of ELP described in the literature are based on the extended repetition of the bovine pentapeptide elastin motif. Human elastin-like polypeptides (HELP) [6, 8, 23, 25] have different structural domains with alternating hydrophobic and hydrophilic, rich in lysine, domains, responsible for the self assembly of the polypeptides and facilitating the physiological crosslinking in fibrillar polymeric structures that gives elastin its extensibility and elastic recoil properties.

In this work, enzymatically cross-linked matrices were developed with a genetically engineered HELP [40] for potential vascular application. The HELP studied in this work was designed to genetically encode domains containing glutamine and lysine-residues which can undergo enzymatic crosslinking by transglutaminase (TGase) (Scheme 1). TGase was previously employed for the crosslinking of ELP to create in situ cross-linkable, cell loaded, hydrogels for functional cartilage repair [19] and to produce collagen-ELP gels, with the aim to improve proteolytic resistance and increase mechanical strength of collagen based scaffold [40]. Recent successful results in the design and production of this HELP polypeptide by recombinant DNA technology in plants (tobacco leaf and rice seed) are opening new perspectives for a large-scale production of the recombinant protein [41].

2 Materials and methods

2.1 Materials

Bacterial transglutaminase (TGase), from *Streptomyces mobaraensis* was supplied by N Zyme (BioTec GmbH). The other chemicals and reagents were purchased from Sigma-Aldrich and used as provided.

MRGSHHHHHHGS
AAAAAAKAAAKAAQFGL VPGVG **VAPGVG VAPGVG VAPGVG LAPGVG VAPGVG**
VAPGVG VAPGIAPA AAAAAAKAAAKAAQFGL VPGVG **VAPGVG VAPGVG**
VAPGVG LAPGVG VAPGVG VAPGVG VAPGIAPA AAAAAAKAAAKAAQFGL VPGVG
VAPGVG VAPGVG VAPGVG LAPGVG VAPGVG VAPGVG VAPGI
AAAAAAAKAAAKAAQFGL VPGVG **VAPGVG VAPGVG VAPGVG LAPGVG**
VAPGVG VAPGIAPA AAAAAAKAAAKAAQFGL VPGVG **VAPGVG VAPGVG VAPGVG**
LAPGVG VAPGVG VAPGVG VAPGIAPA AAAAAAKAAAKAAQFGL VPGVG **VAPGVG**
VAPGVG VAPGVG LAPGVG VAPGVG VAPGVG VAPGI
AAAAAAAKAAAKAAQFGL VPGVG **VAPGVG VAPGVG VAPGVG LAPGVG VAPGVG**
VAPGVG VAPGIAPA AAAAAAKAAAKAAQFGL VPGVG **VAPGVG VAPGVG VAPGVG**
LAPGVG VAPGVG VAPGVG VAPGIAPGV*

Scheme 1 Primary structure of the HELP = $(XY)_8$, where X *crosslinking domain* = AAAAAAKAAAKAAQGFL, and Y *human elastin repeat* = VPGVG (VPAGVG)*$_6$. MW = 45,000. pI ≅ 12. *V* = valine, *A* = alanine, *P* = proline, *G* = glycine, *K* = lysine

2.2 HELP production

The HELP was produced as previously described [40]. Briefly, the fragment spanning part of exons 23 and 24 of the human elastin was amplified by PCR. The recursive directional ligation (RDL) was used to create a synthetic gene that was inserted in an expression vector, pEX8EL. A suitable *Escherichia coli* strain (B121(DE3)pLysS) was transformed with the plasmid construct. Bacteria were grown at 37°C, then collected by centrifugation. After different cycles of centrifugation/redissolution, ~1 g of purified artificial protein was obtained, freeze-dried and its purity was verified by SDS/PAGE electrophoresis [40].

2.2.1 Preparation of cross-linked HELP matrices

To crosslink the HELP with bacterial TGase, the artificial protein was dissolved in 600 µl of a Tris/HCl solution (pH = 8) to a final concentration of 5 and 6% w/v. The solution was stored in an ice bath for 30 min to promote HELP solubilization. Subsequently, 24 µl of a 6% w/v TGase aqueous solution were added and the reaction mixture was quickly mixed. Aliquots (200 µl each) of the solution were placed in PTFE molds (Ø = 5 mm, h = 1 mm) and incubated at room temperature for 3 h. The obtained HELP matrices (HELP5 and HELP6, respectively obtained from 5 and 6% w/v HELP solutions) were removed from the molds and rinsed in ultra-pure water for 30 min to remove the unreacted TGase enzyme. Except for the mechanical and in vitro tests, HELP matrices were freeze-dried (LIO-5P 4K, 5Pascal), after cooling at −20°C, producing HELP5f and HELP6f matrices, respectively.

2.2.2 Scanning electron microscopy

Freeze-dried HELP5f and HELP6f matrices were observed by scanning electron microscope (SEM, StereoScan 360, Cambridge Instruments). The freeze-dried samples were mounted on aluminum stubs, lined with carbon pads and gold coated (Sputter Coater S150B, Edwards). The images were acquired at 100× magnification.

2.2.3 ATR-FTIR spectroscopy

Structural changes in TGase cross-linked HELP5f and HELP6f matrices, in comparison with uncross-linked HELP, were studied by Fourier Transformed Infrared (FTIR) spectroscopy. Spectra were recorded at a resolution of 4 cm^{-1}, in the region of 4000–700 cm^{-1}, using a FTIR spectrometer (Nicolet 6700, Thermo Electron Corporation), equipped with a single-bounce attenuated total reflectance accessory (ATR, HATR Smart, Thermo) with a ZnSe crystal. The ratio ($\nu_{(C-N)}/\nu_{(C-H)}$) between the peak area of the primary C–N stretching (1090–1045 cm^{-1}) and the one attributed to the aliphatic C–H stretching (2940−2840 cm^{-1}) was calculated. Intensity of C–H stretching was assumed constant in the cross-linking reaction. Spectra were elaborated using OMNIC® software.

2.2.4 Water uptake

The swelling rate of freeze-dried HELP samples (Ø = 5 mm, h = 1 mm) was investigated by immersing the dried samples (n = 3) in 10 ml of deionized water for 5, 10, 15, 30 min and, 1, 2, 4, 8 and 24 h at 37°C. At each time-point, HELP5f and HELP6f samples were drawn from the solvent, wiped with filter paper to remove the liquid in excess and weighed. The water uptake (W.U.%) was calculated according to the formula (1), where W_0 is the dry weight, and W_t the wet weight at the time-point t:

$$\text{W.U.}\% = \frac{(W_t - W_0)}{W_0} \times 100 \quad (1)$$

2.2.5 Mechanical compression tests

Compression mechanical tests were performed using a Dynamic Mechanical Analyzer (DMA, model 2980, TA

Instruments), by applying a compression stress at 37°C with a frequency sweep. Briefly, after an isotherm at 37°C for 5 min and a preload of 0.05 N, a compression stress with a 20 μm amplitude was applied, with a frequency sweep in the range 0.5–5 Hz (0.5, 1, 2, 3, 4, 5 Hz), by performing 10 consecutive cycles. The tests were carried out in triplicate using HELP5 and HELP6 samples as obtained from the enzymatic cross-linking and purification in ultra-pure water ($Ø = 5$ mm, $h = 1$ mm), and maintaining the samples in a custom made chamber filled with distilled water for all the test time. Storage modulus (E'), loss modulus (E'') and tanδ (E''/E'), which is a measure of the amount of the energy dissipated as heat during the test, were considered for each test cycle. Statistical analysis (Origin 6.0 software) were performed using a t test (Student test) and a One-way ANOVA test, with significance level $P = 0.05$. Before each statistical test, normal distribution was verified by normal probability plots.

2.2.6 *Cytotoxicity tests*

Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained by enzymatic digestion of umbilical vein endothelial layer with a 0.1% collagenase IV solution (Sigma-Aldrich). The cells were seeded on Petri dishes (BD Biosciences, Franklin Lakes, NJ, USA) previously coated with fibronectin (1 μg/ml; Sigma-Aldrich) and cultured with Endothelial Cell growth Medium MV2 (basal medium, Promocell) supplemented with 5% FCS, ascorbic acid (1 μg/ml), hFGF-2 (10 ng/ml), hEGF (5 ng/ml), hydrocortisone (0.2 μg/ml), R3-IGF-1 (20 ng/ml), VEGF (0.5 ng/ml) (endothelial MV2 medium kit, Promocell), and 1% antibiotic solution (Sigma-Aldrich), containing streptomicine sulfate (10 ng/ml), amphotericin-B (250 ng/ml) and penicillin (100 U/ml). The cultures were incubated at 37°C in a humidified atmosphere. Endothelial cells were used until the 4th passage and harvested at 80% confluence.

To obtain cross-linked HELP matrices for the in vitro cytotoxicity tests, 10 mg of lyophilized pure HELP were solubilized into 200 μl of 10 mM TRIS (pH 8) at 0°C; after that, 50 μl of 60 μg/ml of TGase solution were added to induce cross-linking. The solution (30 μl) was placed on each well of a 96-well plate; after 40 min at r.t. under laminar flow, HELP5 matrices were extensively washed with phosphate buffered solution (PBS; Gibco-Invitrogen Corporation) to remove the excess of transglutaminase. Further washings were carried out after an overnight incubation at 37°C and before the cytotoxicity tests.

The HUVECs (1.5×10^4 cm^{-2}) were seeded into a 96-well plate, and 5.0 μm pore Transwell inserts (Corning Inc.), containing the HELP5 samples, were put on each well ($n = 3$). After 1, 3 and 7 days from seeding, cell proliferation was determined using the CellTite 96® AQueous One Solution Cell Proliferation Assay (Promega), according to the manufacter's instructions. Briefly, HUVECs were treated with 10% MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) for 4 h, and the optical density of the purple formazan produced in living cells was measured at 490 nm, using a Microplate autoreader EL 13 (BIO-TEK Instruments Inc.). The linearity of absorbance of formazan over a range of 3×10^3–20×10^4 cells was established by determining the linear coefficient (0.9858 for HUVECs).

The indirect cytotoxicity was also evaluated by incubating HELP5 matrices in MV2 medium at 37°C for 1, 3 and 7 days. At each time-point, the culture medium was removed and centrifuged. HUVECs (1.5×10^4/cm^2) were seeded into a 96-well plate. After 24 h, the medium was removed and replaced with HELP-conditioned media. After a 24 h incubation period, the MTS dye assay was used to evaluate the cell proliferation, following the same procedure described above.

All the cytotoxicity tests were performed in triplicate and the results were expressed as percentage of cells versus control (Tissue Culture Polystyrene, TCPS).

3 Results and discussion

3.1 Cross-linked HELP matrices preparation

In this study the effect of the cross-linking with the enzyme (TGase) of a novel genetically engineered HELP is described. To study the cross-linking reaction, some experimental parameters (HELP and TGase concentration, buffer, temperature) were purposely varied. The optimal temperature resulted to be 23 ± 1°C, as at higher temperatures (37 and 42°C) protein coacervation, and possibly solvent evaporation, occurred. In addition, the effect of the ionic strength was evaluated, in particular the use of a TRIS buffer at pH = 8, with or without different molarity of NaCl. The addition of NaCl resulted in a slower cross-linking kinetic [42]. The reaction in TRIS buffer at pH 8 was completed within 3 h, and after this time, a gel-like structure was obtained. Figure 1 shows an image of a cross-linked HELP matrix.

Concentrations of the 5 and 6% w/v HELP were selected as optimal concentration for the cross-linking reaction because lower concentration values (2.5, 3, 4% w/v) produced gels with inadequate mechanical properties, whereas concentrations at a higher level (higher than 6% w/v) exhibited solubility problems.

Fig. 1 Image of a cross-linked freeze-dried matrix

3.2 Cross-linked HELP matrices characterization

3.2.1 Scanning electron microscopy

The 3D morphology of freeze-dried cross-linked matrices, prepared with 5 and 6% w/v HELP, was observed by SEM. SEM micrographs showed a homogeneous network, a highly porous nature for HELP5f matrix (Fig. 2a), and the presence of a micro- and nano-porosity for HELP6f (Fig. 2b). The images of both matrices suggest the presence of pore interconnection, even though the percentage of open porosity was not quantified.

3.2.2 ATR-FTIR spectroscopy

The identification of the characteristic IR absorption bands in the structure of not cross-linked HELP and freeze-dried cross-linked HELP matrices (HELP5f, HELP6f) was possible by analyzing the respective ATR-FT IR spectra (Fig. 3). The band at $\sim$3300 cm^{-1} represents the N–H stretching of amine and amide groups; the band at $\sim$1650 cm^{-1} (amide I) is due to the C = O stretching of the CONH group, the out-of-plane C–N stretching and a small contribution from the CC-N deformation; the band at $\sim$1530 cm^{-1}, attributed to amide II, represents the N–H in plane bending and the C–N stretching. No additional functional groups were observed in the spectra of the enzymatically cross-linked matrices, when compared to the spectrum of uncross-linked HELP.

As a consequence of the reaction of the lysine amino groups with the carbonyl groups of glutamine, a decrease of the primary amino groups was expected, and therefore a decrease of the intensity of the IR band attributed to the C–N stretching of the primary amine at 1090/1020 cm^{-1}[43].

By analyzing the zone between 1100 and 900 cm^{-1} in the acquired ATR-FTIR spectra (Fig. 4), a decrease of the intensity of the peak at 1027 cm^{-1} was clearly detectable in the cross-linked HELP matrices. To quantify the cross-linking efficacy of HELP by TGase, the ratio between the peak area of the primary C–N stretching ($\nu_{(C-N)}$ at 1027 cm^{-1}) and that of the C–H stretching absorptions ($\nu_{(C-H)}$ in the zone 2692–3118 cm^{-1}) was calculated for the different samples. As shown in Table 1, a decrease of more than one order of magnitude of the area ratio [$\nu_{(C-N)}/\nu_{(C-H)}$] occurred after the cross-linking reaction in the HELP matrices, compared to uncross-linked HELP.

The use of FTIR to determine the structure and the conformational changes in HELP after cross-linking was previously reported by Garcia Y et al. [35]. In the reported reference, the peak area ratio between the amide I (at 1650 cm^{-1}) and the NH stretching (at 3350 cm^{-1}) was calculated, considering that, upon cross-linking, the intensity of amide I band should increase, whereas that of NH stretching should decrease. This was not verified for our cross-linked HELP matrices, as this particular analysis is sensitive to the moisture present in the sample under IR analysis. In effect, the region in close proximity to 3350 cm^{-1} is also interested by the OH stretching vibrations of water, meaning that the presence of moisture in the samples may affect the peak area integration. Furthermore,

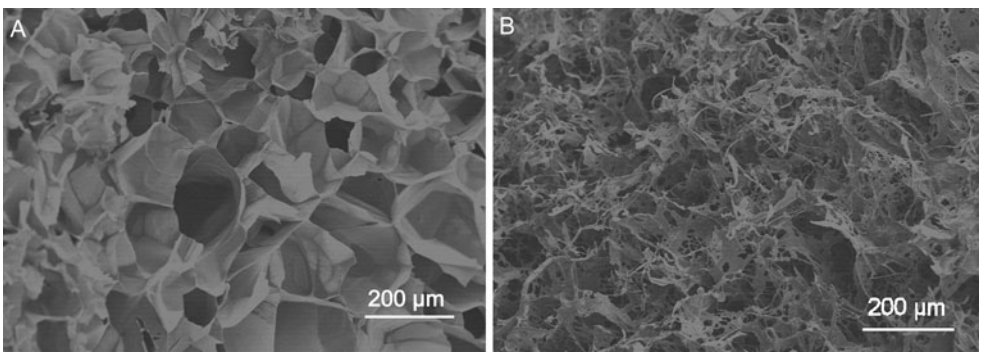

Fig. 2 SEM micrographs of freeze-dried HELP matrices: **a** HELP5f, cross-linked in a 5% w/v concentration, **b** HELP6f, cross-linked in a 6% w/v concentration. *Magnification* ×100

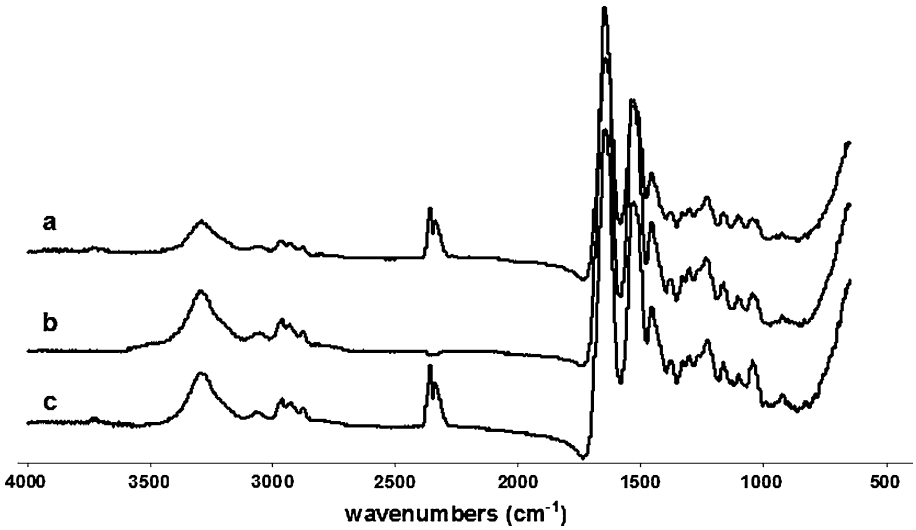

Fig. 3 ATR-FTIR spectra of *a* uncross-linked HELP and cross-linked HELP matrices: *b* HELP5f, *c* HELP6f

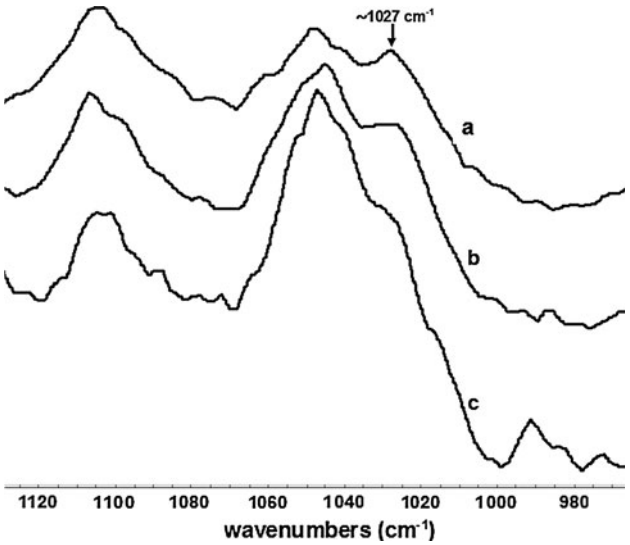

Fig. 4 ATR-FTIR region of the primary amine C–N stretching peak; *a* uncross-linked HELP and cross-linked HELP matrices: *b* HELP5f, *c* HELP6f

Table 1 Peak area ratio between primary amine C–N stretching and C–H stretching bands for uncross-linked HELP and cross-linked HELP matrices

Sample	A_{C-N}/A_{C-H}
HELP	0.458
HELP5f	0.027
HELP6f	0.020

the two bands (amide I and NH stretching) are related to the vibrations of the numerous amide/peptide bonds in the protein structure and only in minimal part affected by the amide bond created by cross-linking.

3.2.3 Water uptake

The water uptake of cross-linked lyophilized HELP matrices was assessed in water at 37°C for HELP5f and HELP6f (Fig. 5). In the case of HELP5f, the maximum water uptake (≈2500%) was reached after 2 and 3 h, and retained up to 24 h (end of the test) with a slight decrease. In the case of HELP6f the maximum water adsorption was higher (≈2700%) and it was reached after 10 min, then a fast decrease was observed and a mean value at about 1000% was reached at longer incubation times with a wide dispersion of the results.

These results appear not to be related to a different cross-linking extent, as in FTIR results (Table 1) the formation of amide bonds are similar. However, this solution concentration affected the morphology of the lyophilized matrices, as shown in SEM images reported in Fig. 2. A higher surface/mass area is observable for HELP6f, with smaller pores and smaller pore walls. The consequent fast swelling can be attributed to capillarity effects in HELP6f. In fact, the behavior of the air-dried samples significantly differ from the lyophilized ones, showing a lower W.U. % (1100 vs. 2450 after 6 h), with a slower kinetic (data not shown).

The fast decrease of water uptake in HELP6f can account a weight loss from the matrices. The dissolution appear to be related more to degradative processes, eased by the surface area titration, than to soluble, uncross-linked, chains.

Water uptake values were of the same order of magnitude to that reported for an α-elastin hydrogel cross-linked with glutaraldehyde at high pressure CO_2 [21, 23] and higher than the ones obtained in the case of elastin-based hydrogels fabricated by use of other cross-linkers.

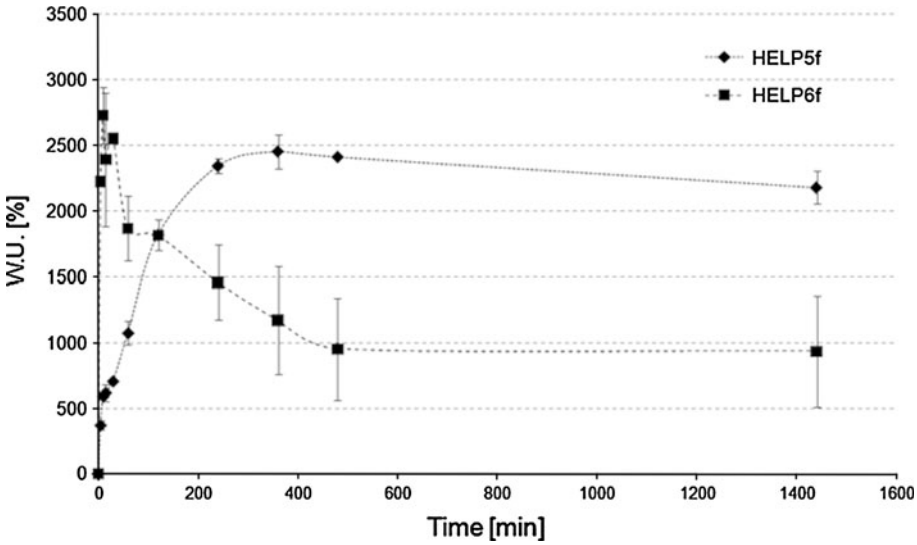

Fig. 5 Water Uptake (W.U. %) in aqueous medium at 37°C of freeze-dried cross-linked HELP5f and HELP6f matrices up to 24 h. Results are expressed as mean ± SD of three independent experiments

3.2.4 Mechanical compression tests

Mechanical compression tests performed at 37°C in a frequency sweep mode indicated, for the cross-linked, not-lyophilized matrices HELP5 and HELP6, values of the storage modulus (E') in the range of 2 and 3 kPa, respectively, and a similar general trend for both materials, indicating no changes in the contribution of the elastic component to the mechanical behavior of the two matrices, by increasing the number of cycles and frequency.

By examining the average values and standard deviation of E' at 1st, 5th, and 10th cycle (Fig. 6a), no significant differences were detectable for HELP5, either considering the E' values at the same frequency ($P > 0.05$) or at the same cycle by increasing the frequency ($P > 0.05$). By observing the E' values of HELP6 matrices (Fig. 6b), a significant difference was detectable only between the frequency of 0.5 Hz and the other tests frequencies ($P < 0.05$); no significant differences were detectable in the E' values recorded at the same frequency for the three considered cycles ($P > 0.05$) and for the same cycle at frequencies higher that 0.5 Hz ($P > 0.05$).

Tan δ (E''/E') values decreased by increasing the test frequency, indicating a major contribution of the elastic component to the mechanical behavior of both HELP matrices at higher frequency values (Fig. 7). A significant difference ($P < 0.05$) was observed between the lowest (0.5 Hz) and highest frequency (5 Hz), whereas no significant differences were evidenced among the three considered cycles ($P > 0.05$) at the same frequency.

On the whole, in comparison with HELP5, higher values of the storage modulus and tan δ were observed for HELP6 matrices, probably due to lower water content deriving from a more concentrated HELP solution.

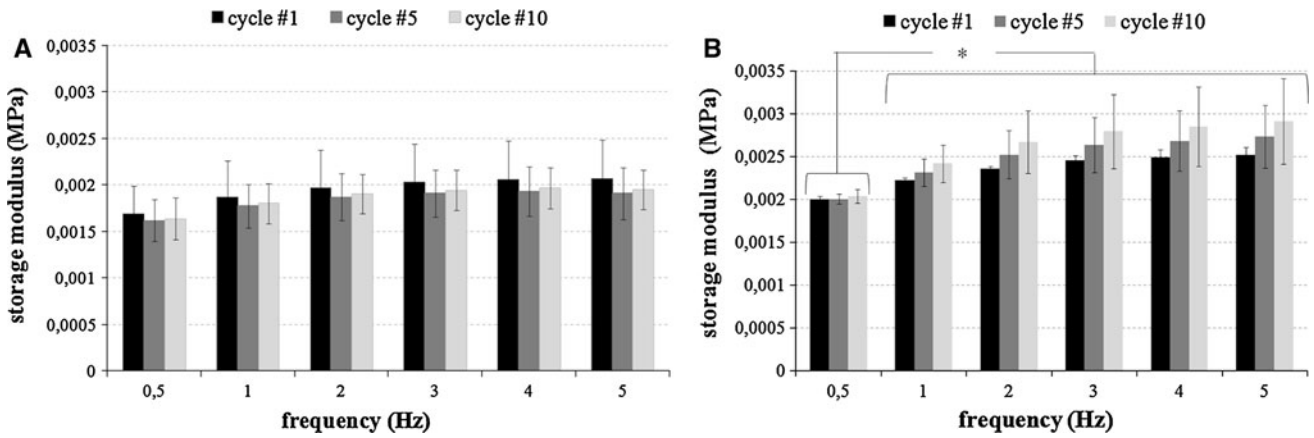

Fig. 6 Results of the compressive mechanical tests (E') performed at 37°C on HELP5 (**a**) and HELP6 (**b**) matrices by applying a compression stress with a frequency sweep. Results are expressed as mean ± SD of the storage modulus (E') at 1st, 5th, and 10th cycle $n = 3$. One-way *Anova* test showed a statistically difference among 0.5 Hz and the other frequencies (*$P < 0.05$), at cycle 1, 5, and 10

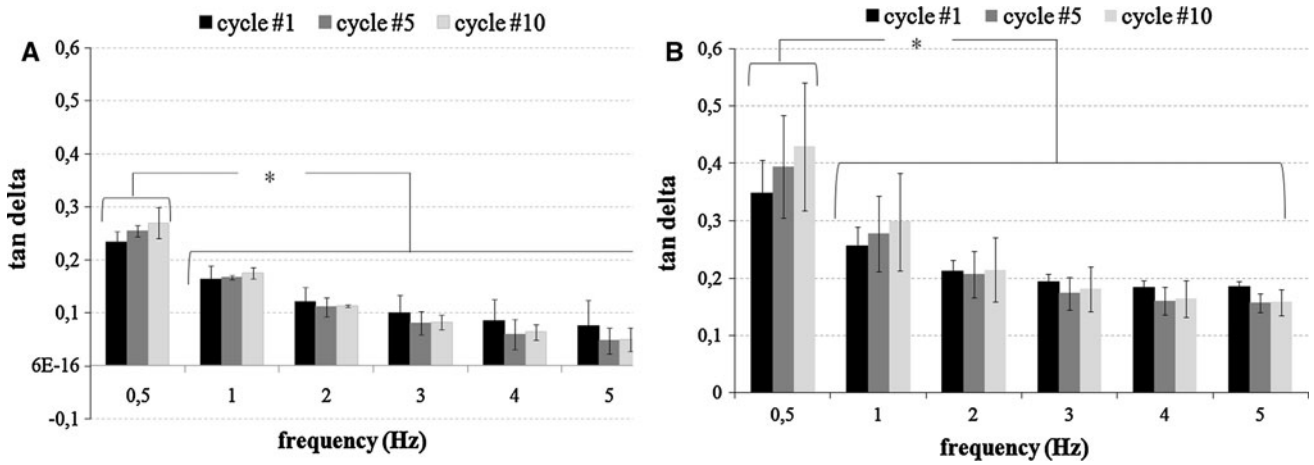

Fig. 7 Results of the compressive mechanical tests (tan δ) performed at 37°C on HELP5 (**a**) and HELP6 (**b**) matrices by applying a compression stress with a frequency sweep. Results are expressed as mean ± SD of tan δ at 1st, 5th, and 10th cycle $n = 3$. One-way *Anova* test showed a statistically difference among 0.5 Hz and the other frequencies ($*P < 0.05$), at cycle 1, 5, and 10

Even though a comparison with the mechanical properties of ELP-based matrices is difficult due to the different test procedure [12, 18, 22, 32], and cross-linker used [32, 44], our data appears in the same range of values. It is worth noticing that no mechanical data related to enzymatically cross-linked human recombinant elastin-based matrices have been previously reported in literature.

3.2.5 *In vitro cytotoxicity tests*

Considering a potential application of HELP matrices for vascular tissue engineering, we have investigated whether the polymer or its degradation products may affect the endothelial cell proliferation. It is well known that the endothelial cell lining is a key factor to maintain vessel patency. Endothelization of luminal surfaces of engineered vessels has been carried out to reduce or avoid thromboembolization [45].

HUVECs were cultured for 1, 3 and 7 days in presence of HELP5 matrices, but separated with Transwell filter chambers (Fig. 8a), or in the presence of the extracts, obtained by conditioning the HELP5 matrices in MV2 culture medium for 24 h (Fig. 8b). As shown in Fig. 8, the percentage of living cells was comparable with the control at each time-point for the two different tests, and therefore no cytotoxicity was observed for HELP5 matrices in these test conditions.

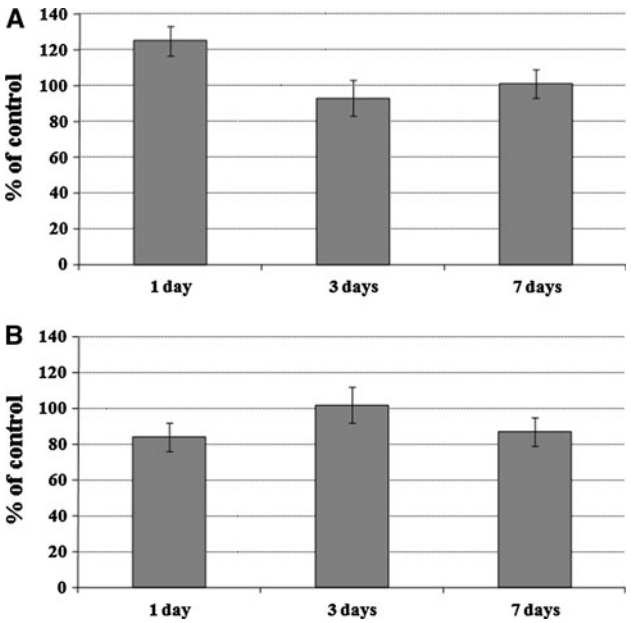

Fig. 8 Results of cytotoxicity assays. **a** HUVECs were seeded on the bottom of culture wells and HELP5 samples were put into Transwell filter chambers; MTS assay was performed after 1, 3 and 7 days of culture. **b** HUVECs were cultured in the presence of MV2 media conditioned with HELP5 samples for 1, 3, and 7 days; 24 h after seeding, cell proliferation was determined by MTS assay. Results are expressed as percent of cells versus control (cell cultures in the absence of HELP5 or HELP5 extracts), as mean ± SD of three independent experiments

4 Conclusions

The cross-linking of the HELP was accomplished through the enzyme transglutaminase, by exploiting *ad hoc* designed insert of cross-linking sites containing glutamine and lysine in the recombinant polymer.

A new method for the evaluation of the HELP cross-linking, based on the infrared spectroscopy analysis, was purposely developed and the peak area of the C–H stretching was used as an internal reference to compare changes of the primary C–N stretching peak area occurring upon cross-linking. The ratio between these two peak areas

resulted sensitive to estimate the cross-linking effect on the polypeptide chemical structure. The swelling ratios of the cross-linked matrices was comparable to the highest reported for elastin hydrogels and the compressive mechanical properties shown by the enzymatically cross-linked HELP hydrogels indicated an elastic behavior with an adequate stability under our test conditions.

Cytotoxicity assays, performed with HUVECs cultured in the presence of cross-linked HELP5 or kept in contact with HELP-conditioned media, demonstrated the absence of cytotoxic effects.

On the whole, our results suggest that the produced HELP-based matrices can be proposed as non-conventional scaffolds for vascular tissue regeneration. This potential application is presently under investigation by in vitro tests carried out by using both HUVECs and human smooth muscle cells. In addition, our recent successful results in the design and production of the HELP polypeptide by recombinant DNA technology in plants (tobacco leaf and rice seed) are opening new perspectives for a large-scale production of the recombinant protein [41], offering new possibilities for a systematic characterization of the cross-linked matrices and for improving this research line.

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