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MICRO-/NANOSTRUCTURED POLYMERIC MATERIALS: POLY(ε-CAPROLACTONE) CROSSLINKED COLLAGEN SPONGES

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A series of biodegradable porous sponges was prepared by using a combination of collagen and poly(ε-caprolactone) in various compositions. To improve the control on the characteristics of the products, hybrid crosslinking methods were applied. Short-range and long-range crosslinking techniques based on physical and chemical crosslinking approaches were combined, including UV irradiation, the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxy-succinimide system, sylanols and of a bifunctional poly(ε-caprolactone) reactive derivative. The new hybrid polymeric materials were characterized by Fourier transform infrared (FTIR) spectroscopy and thermal analysis (DSC). The morphology and swelling behavior of the products were also studied. It was found that matrix features depend on the concentration of poly(ε-caprolactone) derivative used as a long-range crosslinker in the initial formulation and on preparation conditions, especially on UV irradiation duration.

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1. Introduction

Tissue engineering (TE) implies the application of knowledge and expertise from a multidisciplinary field to develop and manufacture therapeutic products that utilize the combination of matrix scaffolds with viable human cell systems or cell responsive biomolecules derived from such cells for the repair, restoration or regeneration of cells or tissue damaged by injury, disease, or congenital defects [1]. To provide an appropriate support for cells mimicking the architecture of extracellular matrix (ECM), the scaffolds or matrices must have a high, prerequisite porosity in order to ensure appropriate structural space for specific cell accommodation and to facilitate efficient exchange of nutrient and metabolic waste between a scaffold and the environment, maintaining at the same time mechanical stability at the defect site of the host. Appropriate scaffolds are thus a key element in developing effective TE products. The development of micro and nanostructured materials, recent additions to materials in relation to tissue engineering, has enhanced the opportunities of fabricating scaffolds able to mimic the architecture of natural human tissues. They offer the possibility of increased control on the porous structure, functionality, biocompatibility and biodegradability in order to favor cell adhesion, proliferation, migration, and differentiation together with appropriate biostability – the desired properties for engineering tissues [2].

Considering these requirements, initially, biodegradable synthetic polymers were proposed for scaffold design, due to their ability to define the required shape and mechanical properties, but they were proved to be unable to provide any biological information to the growing construct [3].

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Consequently, the research interest shifted later to the development of biomaterials of natural origin, which are biointeractive and allow seamless host–graft integration. On the other hand, if natural biopolymers exhibit excellent biological properties, they lack the bio-mechanical features required by specific medical applications. In order to obviate these shortcomings, combinations of synthetic and natural biodegradable polymers became the envisaged new materials, i.e. their corresponding blends, interpenetrating networks or copolymers. In this context, a number of recent papers report on the synthesis and evaluation of biodegradable matrices based on collagen modified by addition of poly(ε-caprolactone) (PCL), intended for the preparation of porous scaffolds. In these systems PCL provides the mechanical stability and collagen supports the adhesion, proliferation and migration of the cells inside the porous scaffolds to form the repairing tissue substitute. Despite their shortcomings, such as poor hydrophilicity resulting in poor cell attachment and proliferation rate characteristic to PCL, and poor mechanical properties and low stability in various physiological solvents in the case of collagen, these polymeric materials can be adapted to successfully meet a range of different clinical applications [3-6] by crosslinking [6, 7], blending with other polymers [5], chemical modification [4], filling with solid inclusions [7]. Among the new materials developed according to these strategies, collagen:PCL biocomposites were especially found to provide a further range of applications in regenerative medicine [8-10]. Their pore characteristics and morphological features can influence cell behavior and are important factors in the development of artificial skin and other tissues.

In this paper, continuing our interest in polymeric biomaterials [11], the preparation and crosslinking of hybrid biodegradable porous sponges based on collagen and PCL was investigated in order to achieve improved, prerequisite performances.

Crosslinking is usually used to stabilize collagen and collagen-based matrices [13]. The (chemical or physical) introduction of the crosslinking agents can modify the biological and physico-chemical properties of the scaffolds**,** also improving the mechanical performances (strength and durability). The crosslinking density strongly affects the biodegradation rate of collagen-based implants, can improve the tensile properties of the materials and may also ensure prerequisite morphological features for specific applications (TE). Both recipe formulation and the crosslinking conditions are highly important in producing collagen-based scaffolds with appropriate mechano-physical, chemical, biological properties and controlled porosity. Different physical and chemical crosslinking methods were developed [17]. The main physical methods, i.e. UV or dehydrothermal (DHT) crosslinking, have the advantage of cleaness but may induce partial denaturation of collagen fibers. The chemical methods are generally divided into two categories – *bifunctional*- (aldehydes, epoxides, isocyanates, alkyl/aryl halides, multifunctional activated synthetic hydrophilic polymers and mixtures thereof) and *amide-type* (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide - EDC/NHS). Some drawbacks are associated with the use of some crosslinking systems, such as a decrease in biocompatility due to the potential toxic effect of residual unreacted or partially reacted crosslinking agent and compounds released when the biomaterial is exposed to biological environments (glutaraldehyde), a decrease in elasticity, toughness and cells biocompatibility accompanying the increase in tensile strength (EDC/NHS system), low coupling efficiency (carbodiimides), possible collagen substrate denaturation and uncontrolled modification (UV and laser treatment), relatively poor control of the final porosity. This is why, during the past few years, research focused on the use of alternative crosslinking agents and techniques. Recently, the use of hybrid crosslinking systems was investigated [18], opening new routes for materials with performances required in biomedical applications.

In this context, a special interest was devoted in the present investigation to the evaluation of the efficacy of poly(ε-caprolactone) diisocyanate as a long-range crosslinker, in combination with short-range crosslinking methods (UV irradiation) and reagents (EDC/NHS system) in yielding hybrid porous matrices with controlled morphology and bio-mechanical characteristics correlated to the recipe formulation and preparation conditions.

2. Experimental

2.1. Materials

0.8% (w/v) acidic solutions (pH~2) of type I atelocollagen (AteCol) were prepared and characterized according to ref. [12]. Methylsilanetriol stabilized on marine collagen hydrolysate microparticles (MSHC) was kindly supplied by the EXSIMOL S. A. M. company (Monaco). Poly(ε-caprolactone) glycols (PCL, Mn-2000), 4,4'-methylenebis-(cyclohexyl isocyanate) (H12MDI), Triton X-100, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (NHS), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, 1M) and dimethylsulfoxide (DMSO) were purchased from Fluka (Germany). All other solvents (methylene chloride, acetone) and reagents of analytical grade were used as received. Bidistilled water was used for all experiments.

2.2. Scaffolds preparation and crosslinking

PCL-DI was prepared by reacting vacuum dried PCL with excess H12MDI, in bulk, at 70 °C for 5h, with stirring under inert atmosphere (nitrogen). Excess H12MDI was removed under high vacuum. The product was then characterized by spectral (^{1}H-NMR and FTIR) and analytical methods and stored in desiccator until use.

Two groups of spongy scaffolds of different formulations were prepared, with different ratios of AteCol, MSHC and PCL-DI. Briefly, porous non-crosslinked AteCol matrices (references) were obtained after lyophilization of an acidic solution (0.8 % w/v, pH~2). The compositions with MSHC were prepared by addition of the appropriate amount of microparticles to the stirred AteCol solution. For the formulations containing PCL, the calculated PCL-DI amounts in the appropriate volume of solvent (2:3 v/v acetone:DMSO) containing 4 wt% (relative to PCL-DI) stabilizer (Triton X-100) were added by dropping to the collagen-based dispersions, the mixtures were homogenized 10 min by stirring and 5 min by sonnication. After deaeration, the mixtures were frozen (-20 °C) and subsequently lyophilized with a CHRIST freeze dryer, Alpha 1-4 LSC type. The sponges were then subjected or not to short-range crosslinking by UV irradiation (Osram HBO 200 W super pressure mercury lamp, 14.6 W, Iv = 1100 cd) for different periods of time, at 15 °C.

For comparison, a third group of samples was prepared by crosslinking AteCol lyophilized sponges using EDC/NHS system in ethanol/water mixture (EDC:NHS:collagen carboxylic acid groups 10:10:1, ethanol mole concentration of 0.13) according to a recently developed alternative [13]. After 14 h reaction at 4 ºC, the scaffolds were washed with saline (15 min), water (3x5min), 0.1 m Na_2HPO_4 (pH 9.1) for 30 min, phosphate buffer (3x30 min), water (3x5 min), citrate buffer (3x30 min). Part of the resulted dispersion was mixed with an appropriate amount of PCL-DI in acetone-DMSO. The prepared mixtures were degassed to remove the bubbles, frozen at -20 ºC, lyophilized and then subjected or not to UV irradiation.

2.3. Characterization

The proton nuclear magnetic resonance (^{1}H-NMR) and Fourier transformed infrared (FTIR) spectra were obtained on an Avance DRX400 (Bruker) spectrometer working at 400 MHz and a Vertex 70 (Bruker) spectrophotometer, respectively. DMSO-d_6 was used as a solvent and TMS as internal standard. FTIR spectra were recorded with the resolution of 4 cm^{-1} in the 400–4000 cm^{-1} range. The amide I spectral region was resolved into its underlying components by a curve fitting treatment based on the calculation of the fourth derivative function using SeaSolve PeakFit v 4.12 software. A linear baseline was always used between 1350 and 1750 cm^{-1}. The position of the band components was fixed, whereas their bandwidths could be adjusted to perform the optimized curve-fitting of the amide I profile. The Voigt areas of the components were evaluated based on the mentioned software.

The free amino group content of native and crosslinked samples was determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay [14, 15]. Collagen samples of 3–5 mg were

incubated for 30 min in 1 ml of a 4 wt % solution of $NaHCO_3$. To this mixture 1 ml of a freshly prepared solution of TNBS (0.5 wt %) in 4 wt % $NaHCO_3$ was added. The reaction was allowed to proceed for 2 h at 40 °C. The solid remaining samples were then rinsed with saline solution to remove unreacted TNBS, and subsequently subjected to freeze-drying overnight. HCl (3 ml, 6 M) was added to the dried weighed samples, the temperature was raised to 60 ºC and then maintained constant until the solid samples were fully dissolved. When the sponge was completely dissolved during the reaction with TNBS, the samples were subjected to the reaction with HCl according the same procedure, then the solutions were extracted with diethyl ether, and a known amount was prepared for further determinations. Thereafter the obtained solutions were diluted with distilled water and the absorbance was measured at 345 nm with a SPECORD 200 Analytic Jena ultraviolet spectrophotometer. The concentration of the reacted amine groups was calculated using the following equation [14]:

$$[NH_2] = (A\ V)/(\varepsilon\ l\ m) \quad (1)$$

where $[NH_2]$ denotes the reacted amine group content [in mol/g of collagen-based sponge]; ε, the molar absorption coefficient of trinitrophenyl lysine ($1.46\ 10^4$ l mol^{-1} cm^{-1}); A, the absorbance; V, the volume of the solution [mL]; l, the path length [cm]; and m, the weight of the sample [mg]. The free amine group contents were calculated by assuming that the uncrosslinked AteCol lyophilized sponge has 100 % free amine groups [15].

The thermal stability of the lyophilized samples was assessed by differential scanning calorimetry (DSC) with a Mettler 851 system over a temperature range of -20 °C to 140 °C. The samples were heated at a constant rate of 5 °C/min in nitrogen atmosphere with an empty aluminum pan as the reference. The endothermic peak value was taken as the denaturation temperature (Td). The corresponding denaturation enthalpy (ΔHD) was calculated with respect to the mass of the sample. To avoid errors due to the effect of water content, the samples were previously maintained in a desiccator at a constant 20 % humidity.

The porosity values of the composite sponges were measured by liquid displacement, methylene chloride being used as the displacement liquid [16].

The surface and cross-section morphologies of scaffolds were observed directly by a scanning electron microscope (SEM - Quanta 200 apparatus, working in low vacuum mode) without sputter coating by conducting matter.

The swelling capacity studies were performed at room temperature by immersing the weighed lyophilized samples (three specimens for every formulation) of 2 x 2 x 0.3 cm in bi-distilled water. At specified time intervals the samples were taken out of the water, blotted to remove surface water and weighed. The mass swelling ratio (SR) is calculated by the relation

$$SR\ (g\ g^{-1}) = (Ws–Wd)/Wd \quad \textbf{(2)}$$

where Wd is the weight of dry sample, and Ws is the weight of the swollen one.

3. Results and Discussion

To generate materials able to optimally meet the criteria for scaffolds with controlled characteristics, a hybrid crosslinking methodology was applied to the different collagen-based compositions, namely, a combination of short- and long-range crosslinking, physical and chemical methods. As shown in Table 1, UV irradiation, EDC/NHS system, sylanols and a bifunctional PCL reactive derivative are among them.

Table 1: Collagen based materials formulations

Code		AteCol	Second component		Crosslinking method		
		wt%	Nature, code	wt%	EDC/NHS	UV min	PCL-DI wt%
AteCol		100	-	-	-	0	0
$AteColP_5$						0	5
$AteColP_5$-10						10	
$AteColP_5$-20						20	
$AteColP_5$-30						30	
$AteColP_{15}$						0	15
$AteColP_{15}$-10	I					10	
$AteColP_{15}$-20						20	
$AteColP_{15}$-30						30	
$AteColP_{30}$						0	30
$AteColP_{30}$-10						10	
$AteColP_{30}$-20						20	
$AteColP_{30}$-30						30	
$AteCol_{EN}$		100	-	-	+	0	0
$AteCol_{EN}P_2$	II					0	2
$AteCol_{EN}P_2$-30						30	2
AteCol-SiCol		30	MSHC SiCol	70	-	0	0
AteCol-SiCol-30						30	0
AteCol-$SiColP_1$-30	III					30	1
AteCol-$SiColP_{20}$						0	20
AteCol-$SiColP_{20}$-30						30	20

Short-range crosslinking with carbodiimides and by UV irradiation were selected because they are viable strategies for the preparation of non-toxic collagen-based biomaterials. Silanols were recently introduced, especially in cosmetics formulations, for their peculiar abilities in binding polysaccharides and glycoproteins, as for as for their biological activity (cytostimulation, tissue restructuration, cytoprotection, metabolic modulation), characteristics of interest for TE (i.e. skin substitutes).

FTIR examination in combination with the determination of the residual amino group amount and of the denaturation temperature have been used to follow the changes in polymer matrix structure during the processing to form the 3D matrix.

FTIR spectra evidenced the presence of collagen and PCL in the hybrid matrices, as well as the modification of functional groups involved in the crosslinking reaction (Fig. 1). The main IR bands associated with collagen were mostly attributed to various types of amide bonds [19, 20]. There are nine such bands, called amides A, B, and I–VII (decreasing wavenumber order). They are situated at about ν 3320 (amide A) and ν 3070 (amide B), ν 1656 (amide I), ν 1550 (amide II), ν 1238 (amide III), and ν 1075 cm^{-1} and are mainly attributed to N–H, C=O, N–H, C–N and N–H stretching, and O–H bending, respectively. Absorption features at 1450, 1399, 1339, 1282 and 1203 cm^{-1} are attributed to CH_2 and CH_3 wagging and deformation, and C–N stretching of collagen. Fig. 1 shows the mid-infrared spectra (4000–600 cm^{-1}) of a hybrid sponge with AteCol-$SiColP_{20}$ formulation, before and after UV irradiation, as compared to non-crosslinked AteCol and DSHN (SiCol), as control samples. The spectral changes for various materials are obviously related to their composition and applied crosslinking procedure. By moving from non-crosslinked AteCol toward the highly crosslinked hybrid AteCol-$SiColP_{20}$-30, the spectrum shifts upward toward higher absorption intensities, more pronounced at collagen amide characteristic peaks, i.e. amide A, and amide I, an increasing amount of NH_2 being converted to NH. The new absorptions in the FTIR spectrum of AteCol-$SiColP_{20}$, situated at 2933, 2262, 1724, 1020 and 951 cm^{-1} were related to the inclusion of PCL-DI, being attributed to CH_2, N=C=O, ester carbonyl, and C-O-C

stretching, respectively. By irradiation, the reaction of PCL-DI with collagen NH_2 groups is completed. Thus, the signal from 2262 cm^{-1} is stepwise vanishing and finally disappearing and the absorption intensity of the amide A band is strongly increasing, due to the formation of urethane groups, while the features of the other bands are only slightly changed.

However, a further study of the effects on exposure of hybrid matrices to UV revealed modifications of the physical properties of the other two groups of samples with irradiation duration, as also evidenced by FTIR investigation. Thus, for the first group, comprising samples based on AteCol only, crosslinked with PCL-DI and subjected to different irradiation intervals, it was observed that after 10 min the -N=C=O group absorption is disappearing, while the intensity of amide I and II bands is increasing, followed by a gradual decrease with increase of irradiation duration (Figs. 1 and 2A), in agreement with literature data [21]. For samples having a high content of PCL in their formulations, but no silanol, i.e. the first two groups in Table 1, the signals situated at 2933 and 1724 cm^{-1}, assigned to the CH_2 groups in the polymer matrix and to the ester carbonyl stretching in PCL chain, are strongly diminishing after longer irradiation periods (Figs. 1B, 2B). These aspects were associated with a photodegradation process occurring for more than 10-15 min irradiation, favored by the presence of ester carbonyl groups of PCL, resulting by bond cleavage in carbon dioxide emission and random PCL chain fragmentation, correlated with the evidenced loss of ester linkages. The effect was confirmed by analysis of such samples after rinsing with water, when a significantly decreased absorption was registered at 1020 and 951 cm^{-1}, related to C-O-C stretching in PCL, clearly indicating a chain scission. It is to note that some literature data point also the decreased photostability of collagen when combined with synthetic polymers able to enhance UV absorption [22]. On the other side, the present results suggest that the inclusion of silanols in the formulations may induce an increased photostability of the corresponding systems, while preserving the envisaged properties.

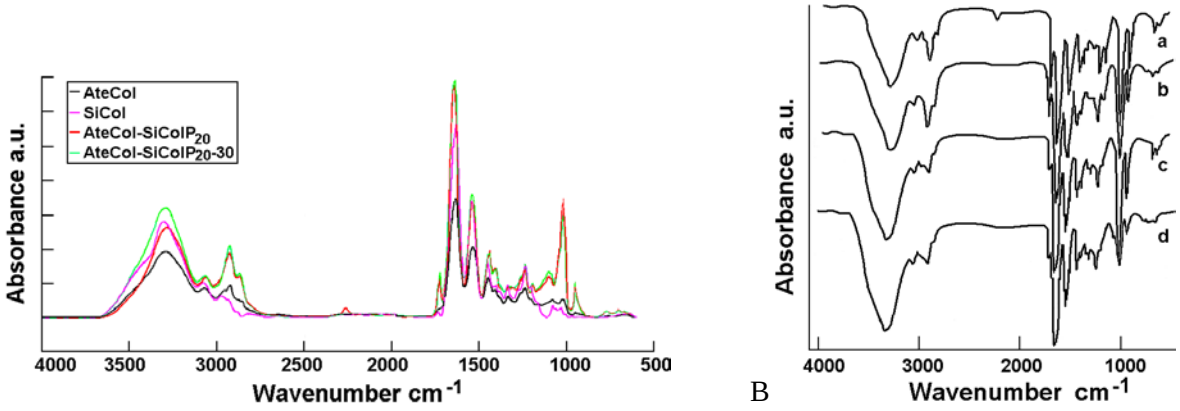

Fig.1. Typical FT-IR spectra of crosslinked collagen-based sponges A: AteCol-SiColP$_{20}$ before and after UV irradiation comparative to non-crosslinked samples (AteCol, SiCol) B: AteCol$_{EN}$P$_2$ before (a) and after UV irradiation for 30 min (b) or 70 min (c), followed by water rinsing (d)

As can be observed from Fig. 2B, the spectra of AteCol$_{EN}$P$_2$ samples, irradiated or not, show a Δν (νI - νII) value higher than 100 cm^{-1} (i.e.107 cm^{-1}), while this difference is of 81 cm^{-1} after the removal of PCL fragments by water rinsing and of 85 cm^{-1} for AteCol$_{EN}$. This is usually correlated with a denaturation of the α-helix of the collagen molecule, here attributable to modifications involved by the inclusion of PCL chains in the rigid, dense network of EDC/NHS crosslinked AteCol.

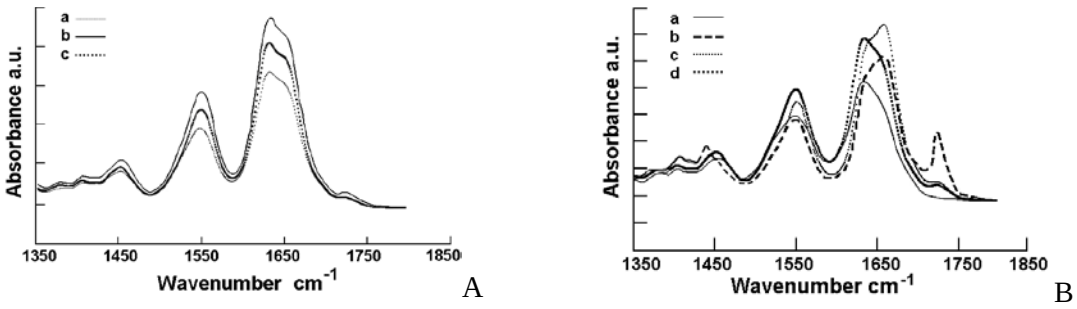

Fig. 2. UV-irradiation effect A: sample AteColP$_5$ before (a) and after UV irradiation for 10 min (b) and 20 min (c); B: samples AteCol (a) and AteCol$_{EN}$P$_2$ before (b) and after 30 min UV irradiation (c), followed by water rinsing (d), respectively

A curve fitting treatment of the 1700–1480 cm^{-1} spectral domain was carried out to quantitatively estimate the relative proportion of each component representing a type of secondary structure (Fig. 3). Two underlying bands in the amide I spectral region, one at 1660 cm^{-1} and another at 1690 cm^{-1}, have been recognized previously as constituent bands useful for FTIR analysis of modifications in collagen [20]. The former has be assigned as representative of triple helices, and the latter has been assigned to "free" carbonyl groups, the relative percent area ratio of the two sub-bands being related to collagen crosslinks. The values of the corresponding ratios for the here prepared samples are given in Table 2. They are in agreement with the calculated crosslinking degree according to TNBS assay results.

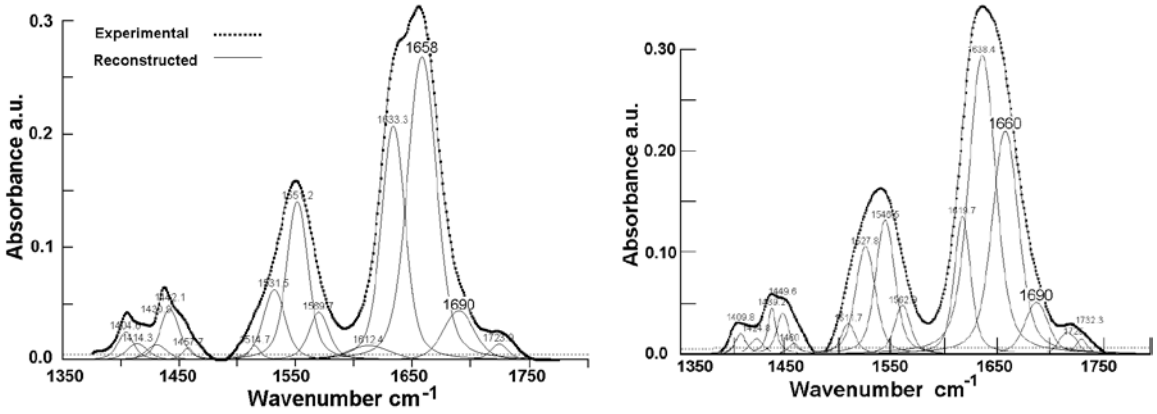

Fig.3. Curve-fitting analysis of the amide I profile in FTIR. Samples: (a) AteCol$_{EN}$P$_2$-30 and (b) AteCol-SiColP$_2$

Table 2. Crosslinking characteristics of collagen-based matrices

Code	**FT-IR**		**TNBS assay**
	A_{1660}/A_{1690}	**A_{1240}/A_{1450}**	**Crosslinking degree %**
AteCol	9.76	0.95	0.0
AteColP$_5$	8.95	0.97	6.7
AteColP$_5$-10	7.66	1.00	29.0
AteColP$_5$-20	5.45	1.00	35.0
AteColP$_5$-30	4.98	1.04	40.0
AteColP$_{15}$	8.12	1.08	12.5
AteColP$_{15}$-10	5.76	0.92	40.3
AteColP$_{15}$-20	4.96	0.85	46.0
AteColP$_{15}$-30	4.92	1.04	45.0

Code	FT-IR		TNBS assay
	A_{1660}/A_{1690}	A_{1240}/A_{1450}	Crosslinking degree %
$AteColP_{30}$	7.71	1.03	18.1
$AteColP_{30}$-10	5.76	1.12	45.0
$AteColP_{30}$-20	4.93	1.16	53.7
$AteColP_{30}$-30	4.67	1.25	51.3
$AteCol_{EN}$	7.25	0.84	66.1
$AteCol_{EN}P_2$	5.67	1.01*	71.0
$AteCol_{EN}P_2$-30	5.41*/5.75**	1.33*/0.96**	73.3*/70.0**
SiCol	5.09	0.72	15.2
AteCol-SiCol	4.91	0.88	8.0
AteCol-SiCol -30	4.55	0.77	25.0
AteCol-$SiColP_{20}$	3.71	0.88	68.0
AteCol-$SiColP_{20}$-30	3.40	0.93	66.3
AteCol-$SiColP_1$-30	5.10	0.85	63.4

* before water rinsing, ** after water rinsing

The absorbance peak at 1238 cm^{-1} is affected by changes in the triple helical structure of collagen and can therefore be used to quantify the denaturation of a collagen sample. The ratios $AIII/A_{1450}$ values are close to unity for most matrices, confirming the preservation of the helical structure of collagen in the hybrid collagen-PCL scaffolds (Table 2). Its slight increase with the increase of UV-irradiation duration for the samples with a high PCL content ($AteColP_{30}$), as well as for $AteCol_{EN}P_2$-30 sustain the previous remarks.

The preservation of the triple helical structure of collagen was also checked by DSC. The degree of crosslinking of the collagen is related to the increase of the denaturation (shrinkage) temperature (Td). At Td, the collagen undergoes an endothermic transition that represents the melting of the triple helical structure of the collagen and thus is a measure of the structural stability of the proteins. A higher helix-coil transition temperature of collagen suggests that additional intermolecular crosslinking of collagen fibers occurs. Thus, the crosslinking density has a dramatic effect on the observed transition temperature, as shown in Fig. 4 and Table 3, where DSC curves and the measured values of Td and of the heat of denaturation (ΔHD) are presented for some representative synthesized samples. The Td values are increasing after crosslinking from 70.8 °C in non-crosslinked AteCol lyophilized sponge up to 84 °C, depending on the type of short-range crosslinking method, long-range crosslinking reagent concentration and duration of UV irradiation, for the irradiated specimens. The high Td value for AteCol-SiCol based group as compared to the AteCol-based one is related to the stabilization by sylanols, the content of DSHN microparticles in the mixture being as high as 70 %. The relative low values (considering the FTIR and TNBS assay data) for the group of samples using the short-range crosslinking EDC/NHS system are most possible related to the use of ethanol in the preparative steps before lyophilization [23].

The DSC investigation also proved the heterogeneous stability of the hybrid matrices in the solid state. The samples without PCL or having a low content of PCL (≤ 2 wt%) showed a single large peak in their DSC thermogram, situated in the 72.7 - 80.9 °C range (Table 3), with a full peak width at half height (fwhh) of ~50 °C (Fig. 4). When PCL-DI was used as a long-range crosslinking agent, depending on concentration and sample treatment, two supplementary peaks, a sharp peak or a shoulder appear in the 46 - 56 °C range (PCL melting temperature). The two melting peaks are attributable to different crystalline domains, in the non-irradiated samples both free and collagen linked PCL chains being present. The broad shoulder appearing for longer irradiation periods may be attributed to the formation of small domains, with a low degree of perfection and partial melting of the defective crystals, originating in PCL chains cleavage and partial degradation.

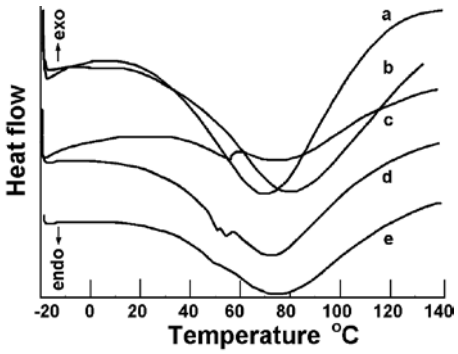

Fig. 4. DSC scans of collagen matrices: (a) AteCol; (b) AteCol$_{EN}$; (c) AteCol$_{EN}$P$_2$-30; (d) AteColP$_{30}$; (e) AteColP$_{30}$-30

Table 3. Thermal and morphological characteristics of the prepared collagen-based matrices

Sample Code	Td °C	Tm °C	ΔHD J g^{-1}	Porosity %	Pore size μm
AteCol	70.78		-279.66	98.4	154/190
AteColP$_5$-30	77.15	49.92 peak	-136.21	94.0	78/110
AteColP$_{15}$-30	77.55	48.47, shoulder	-295.64	93.9	80/120
AteColP$_{30}$	72.22	49.39; 53.03, peaks	-285.27	94.0	60/70
AteColP$_{30}$-30	78.67	47.15, shoulder	-255.96	91.2	100/152
AteCol-SiCol	80.91		-274.87	94.9	74/74
AteCol-SiColP$_{20}$-30	83.91	55.99	-130.99	91.9	65/72
AteCol$_{EN}$	72.74		-318.30	95.6	49/150
AteCol$_{EN}$P$_2$	75.47		-358.80	92.8	255/325
AteCol$_{EN}$P$_2$-30	75.65		-260.20	94.9	354/376

The effect of crosslinking on matrix morphology was investigated. The SEM results showed that the pore size and shape vary (Table 3, Fig. 5) after croslinking, the structural features changing depending on the applied procedure. Basically, the prepared materials exhibit a heteroporous morphology with interconnected pores. The initial, known fibrillar structure of collagen is changing due to fibrils ensheeting/ensheathing in PCL connecting walls. At low PCL content fibrous structures are more pronounced, while integrally lamellar aggregates appear where PCL amount increases. With pore walls increase, the mechanical strength of collagen gels is expected to increase, too. A lamellar structure with smaller, tight, long pores is obtained by using the EDC/NHS system, but this structure is completely changed by addition of PCL-DI followed by irradiation of the lyophilized sponge. The partial photodegradation of PCL bridges at UV irradiation periods longer than 10 min is evidenced by the increase of matrix porosity (Table 3), pore size and their interconnectivity (Fig. 5, c relative to b and g, h to f). Increased microstructure uniformity may be obtained by inclusion of DSHN microparticles, when a hard cellular structure with relatively spherical pores results. Meaningful, the correlation between PCL content/crosslinking procedure with network structure implies a relative control of the matrix properties, allowing a design control for envisaged applications.

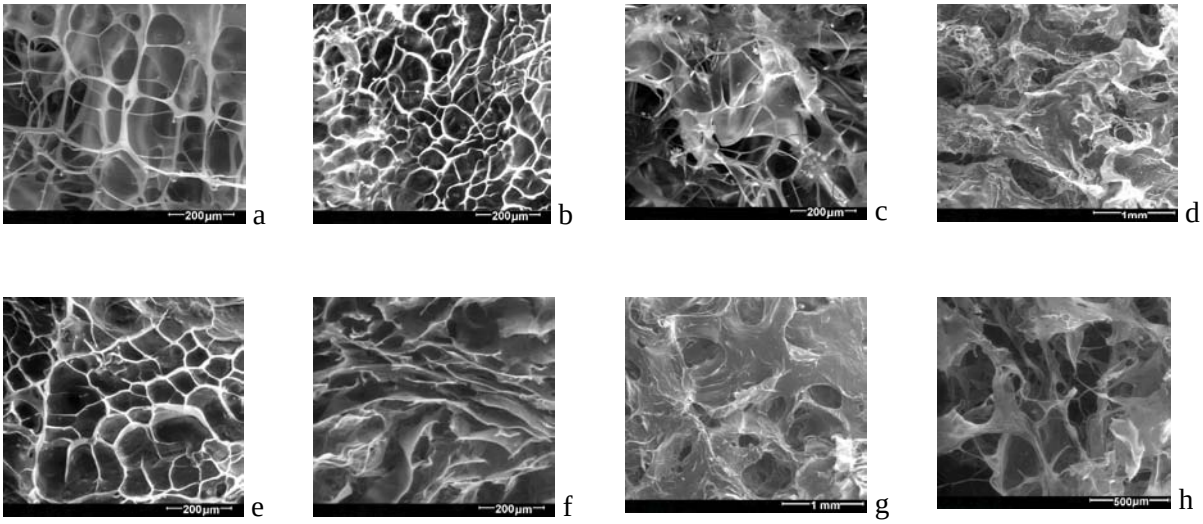

Fig. 5. SEM micrographs of the porous collagen-based structures: (a) AteCol; (b)AteCol$_5$; (c) AteCol$_5$-20; (d) AteCol$_{15}$-30; (e) AteCol-SiColP$_{20}$-30; (f) AteCol$_{EN}$; (g) AteCol$_{EN}$P$_2$; (h) AteCol$_{EN}$P$_2$-30

The equilibrium swelling behavior of the prepared collagen-based materials as a function of time was monitored gravimetrically at 25 °C. As expected, the equilibrium swelling ratio (ESR) values decrease with PCL-DI amount increase in the recipe or by increasing the UV irradiation period (Fig. 6).

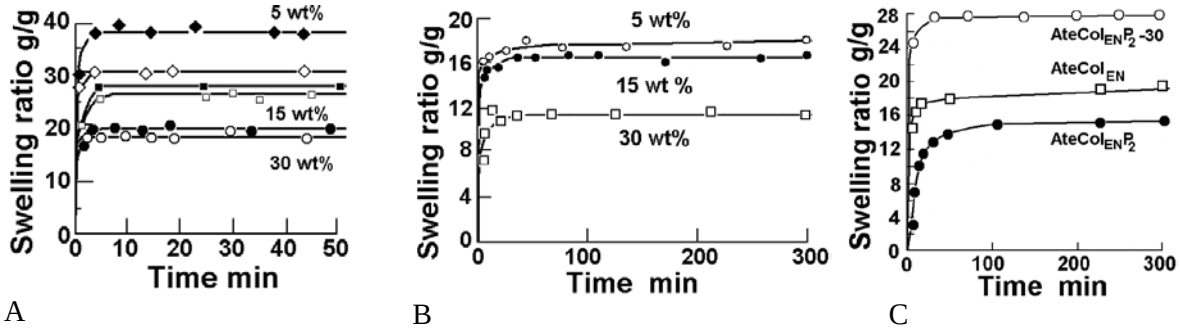

Fig. 6. Swelling kinetics for different matrices formulations (Table 1) and UV irradiation intervals Group I of samples (AteCol crosslinked with various PCL-DI amounts) irradiated for 10 (A-dark), 20 (A-light) and 30 min (B) and (C) group II of samples – AteCol crosslinked by EDC/NHS system with/without PCL-DI

In agreement with the earlier observation of a possible photosensibilization during UV irradiation, after UV exposure for 30 min the equilibrium swelling ratio of AteCol$_{EN}$P$_2$ increased and AteCol-SiColP$_1$ was disintegrated in small fragments. This evidences the importance of UV irradiation interval and also of PCL-DI concentration. A low PCL-DI amount ($\leq$ 1%) makes crosslinking difficult, yielding sponge integrity depreciation.

4. Conclusions

Different collagen/PCL compositions were prepared by a hybrid crosslinking alternative based on the use of PCL-DI as a long-range crosslinking agent and different short-range crosslinking alternatives. The characterization of the lyophilized crosslinked sponges as compared to the non-crosslinked control samples evidenced that PCL-DI acts as an efficient croslinking

agent allowing the generation of hybrid natural/synthetic biodegradable, porous matrices, with improved stability, design and properties controllable from preparation conditions and recipe composition, while preserving the molecular structure and mechanical integrity of collagen. The results point on the possibility to control the final properties by varying these parameters, opening new ways for the generation of scaffolds with prerequisite characteristics, according to the envisaged uses. Such materials could be designed for applications in biomedical (TE scaffolds, wound dressing) and cosmetics areas.

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