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Synthesis and Characterization of Protected Oligourethanes as Crosslinkers of Collagen-Based Scaffolds

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Abstract

This paper describes the preparation and characterization of water-soluble urethane oligomers bearing protected isocyanate groups. It also points out its ability to crosslink decellularized pericardium, as a model collagen scaffold, and to adjust their structural characteristics. A library of oligourethanes was synthesized by varying the molecular weight (Mw 400, 600, 1000 or 2000 g mol^{-1}) of the poly(ethylene glycol) and type of aliphatic diisocyanate (isophorone diisocyanate/IPDI or hexamethylene diisocyanate/HDI). ^{1}H and ^{13}C NMR, FTIR and mass spectrometry demonstrated that the crosslinkers are composed of chains with carbamoylsulfonate end groups that have trimeric and pentameric oligourethanes, and monomeric diisocyanate. The degree of crosslinking and hence the *in vitro* degradation susceptibility of the decellularized pericardium were inversely related to the Mw of the oligourethanes. The toxicity of the extractable products from oligourethane/collagen materials toward fibroblasts and macrophages was found lower for the crosslinker derived from IPDI that for those derived from HDI. On the other hand, the resistance to collagenase or oxidative degradation of the bovine pericardium crosslinked with HDI/oligourethane was higher than the one prepared with IPDI/oligourethane. As the Mw of the oligomers regulates the degree of

crosslinking while the chemical composition influences the cytocompatibility and biodegradation of decellularized pericardium, these urethane oligomers can be used as safer crosslinkers for other protein-based biomaterials.

Keywords: collagen crosslinking; isocyanate blocking; oligourethanes

1. Introduction

Decellularized extracellular matrices have found many tissue engineering applications for example either as dry or hydrated sheets in the treatment of skin wounds. [1-2] These and other applications are based on its composition, structure and mechanical properties but they can be further improved, and their applications expanded, by chemical modification of the macromolecules that constitute the decellularized matrices, including collagen. In this regard, chemical crosslinking is a process intended to slow or prevent degradation of collagen scaffolds, to inhibit the recognition of surface epitopes by the host, and to provide improved mechanical properties for load bearing applications. [3] For instance, crosslinked collagen scaffolds derived from decellularized matrices, such as pericardium, dermis, intestine, gallbladder, etc., have been proposed as meshes in the abdominal wall reconstruction. [2,4-8]

Several crosslinking methods have been used in order to obtain the optimal process to stabilize the collagen and other protein-based biomaterials. Among them, the crosslinking of protein biomaterials by diisocyanate chemistry is an idea widely explored. The crosslinking of protein-based biomaterials with monomeric and macromolecular compounds with isocyanate end groups [6,9-16] have shown favorable effect on biocompatibility when compared to glutaraldehyde (GA) crosslinking. [6,14] The use of hexamethylene diisocyanate (HDI) as a crosslinker for dermal or pericardial collagen was reported first using isopropanol [11] or Tween 80 surfactant [12] because HDI is only slightly water-soluble. HDI and ethyl lysine diisocyanate have been used in the preparation of gelatin-based hydrogels by using poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) [13] or saponin [15] to improve the solubility of diisocyanates in water. The use of surfactants in water or dimethylsulfoxide replacing water [11,15-16] is thought to form micelles that incorporate the crosslinker, thereby shielding the crosslinker from water. [15] On the other hand, the use of poly(ethylene glycol) (PEG)-

derivatives to achieve the chemical crosslinking of proteins has been explored as a strategy to engineer, for examples, hydrogels for tissue sealing/bioadhesion [17] or nanocarriers for drug delivery. [18] The end diol groups of PEG can be functionalized easily with a variety of groups such as acrylates, acrylamide, isocyanates, thiols, maleimides, etc., in order to react with chemical groups in a protein. [19-21]

Crosslinking has an influence on porosity, ultrastructure and surface chemistry, which along with degradation rate represent key factors to disrupt wound contraction, reduce scar formation, and ultimately enhance the scaffold-induced regeneration of skin and peripheral nerves. [8, 22-23] In this sense, the type of crosslinking agent, time, temperature or pressure are variables that allow the adjustment of the density of crosslinking and consequently, the degradation half-life of collagen scaffolds can be fine-tuned. [23] In our earlier work [10], we reported the use of non-catalyzed water-soluble blocked polyurethane prepolymer (PUP) as a bifunctional crosslinker for collagenous scaffolds that allows control of the degree of crosslinking via the process parameters (pH, volume, concentration). The collagen network was coated with PUP, which increased its resistance to collagenase digestion but retained its porosity and showed tensile properties comparable to the GA-treated one. In addition, crosslinking chemistry has been related to differences in the biological response to collagen scaffolds. [6-8] Differences in the chemical composition of collagen scaffold crosslinked with either HDI or GA have been correlated with anti or pro inflammatory milieu in the subcutaneous implant.[6] This in turn produced degradation of GA/collagen by collagenolytic activity and phagocytosis by macrophages. Bearing this in mind, the ambient chemistry and the crosslinking density appear to be crucial in order to tune-up the structural features of collagen scaffolds and to up-regulate their biological performance.

Thermoplastic polyurethanes are commonly synthesized by a two-step polymerization, in which firstly a macrodiol such as PEG diol is reacted with an excess of a diisocyanate in the so-called prepolymerization reaction. The formed oligourethanes or macrodiisocyanates are

subsequently extended with bifunctional molecules to produce the thermoplastic polyurethanes. Alternatively, the hydrophilic macrodiol-based prepolymer can be reacted with a suitable blocking agent to produce a water-soluble product. [24-26] The reaction between the isocyanate group and a bisulfite salt allows the hydrophilic blocking of the isocyanate functionality.[25] So, blocked oligourethanes can offer an advantage due to the high water solubility and suitable permeation behavior of the crosslinkers into scaffolds. When the terminal groups of the resulting products undergo de-protection, the isocyanate groups are regenerated, and the bifunctional product can be used for the reaction with the chemical groups in a protein to achieve crosslinking. Hence, PEG-derived water-soluble oligourethanes bearing protected isocyanates could be used for reaction systems with aqueous media without need of organic solvents or surfactants. This sort of oligourethanes could be applied to a wide range of medical applications such as crosslinkers in the manufacturing of surgical collagen implant for abdominal wall defects or liquid precursors of multicomponent hydrogels. However the effect of the chemical structure of oligourethanes on the density of crosslinking of collagen-based biomaterials has not been investigated. Therefore, the aims of the present study were (i) to synthesize and characterize a library of water-soluble oligourethanes that differ in chemical structure and (ii) to investigate the effect of these oligourethanes on the degree of crosslinking of a collagen scaffold, their *in vitro* degradation and cytotoxicity. To achieve this, bovine pericardium, mainly composed of type I collagen, was selected as biological matrix not only because its well know range of medical applications but also as it retains its ultrastructure, mechanical anisotropy and tensile properties after decellularization. [27] The oligourethanes, to test, were obtained by the reaction of PEG of 400, 600, 1000 or 2000 g mol^{-1} with an excess of hexamethylene diisocyanate or isophorone diisocyanate and by using sodium bisulfite as blocking agent.

2. Experimental Section

2.1. Materials

Poly(ethylene glycol) (PEG, Mw 400, 600, 1000 or 2000 g mol^{-1}), hexamethylene diisocyanate (HDI) isophorone diisocyanate (IPDI), sodium bisulfite, disodium piperazine-1,4-diethanesulphonate hydrate (PIPES), 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) and collagenase type I were acquired from Sigma-Aldrich. Ninhydrin (2,2-dihydroxy-1,3-indanedione) was purchased from Merck. The collagen scaffolds were prepared by decellularization of bovine pericardial tissue using a method, we previously reported it, that combines reversible alkaline swelling and nonionic detergent.[27] Briefly, samples were placed in distilled water (4°C, 18 h) containing calcium oxide (0.4%) and Triton X-100 (0.2%). Samples were stirred (RT, 20 min) in solution containing ammonium sulfate (2%) and then stirred (4°C, 48 h, 20 rpm) in sterilized and filtered PIPES buffered saline solution (PBS, pH 7.4) containing Triton X-100 (1%) and finally washed with PBS.

2.2. Synthesis and characterization of blocked-oligourethanes

2.2.1. Synthesis of oligourethanes

The oligourethanes were synthesized, as previously described elsewhere [10], in bulk in a 100 ml two-necked round bottom flask. The first step in the preparation of the blocked oligourethane was the formation of the prepolymer. Thus, melted PEG was reacted with the diisocyanate in a molar NCO:OH ratio of 4.0:1.0 for 2h at 100°C. At the end of this stage, the temperature was reduced to 60°C. In a second step, sodium bisulfite (40w% solution in water) was added onto the prepolymer and the reaction was continued for 2 h at 40°C. The solution was diluted with water to give a product containing about 30% solids by weight.[28] Blocked-

oligourethane solutions were cooled to room temperature, characterized by ^{1}H, ^{13}C NMR, FTIR, mass spectrometry and differential scanning calorimetry and used as crosslinker of the collagen scaffolds. Table 1 summarizes the designations of the synthesized products with PEG of four molecular weights and two different aliphatic diisocyanates.

Table 1. **Synthesized urethane oligomers**

PEG molecular weight (g mol^{-1})	Diisocyanate	Product designation
600	HDI	H-6
1000		H-10
2000		H-20
400	IPDI	I-4
600		I-6
1000		I-10

2.2.2. Characterization of oligourethanes

FTIR – Infrared spectroscopy information of the blocked oligourethane was obtained using the ATR technique on a Perkin Elmer Spectrum 100 spectrometer. The solutions of the blocked oligourethane were dried in an oven at 40°C under vacuum. The dry samples were deposited on the diamond crystal and ATR-FTIR spectra were recorded at 4 cm^{-1} of resolution in a spectral range from 4000-650 cm^{-1}. Spectra were the average of 32 scans.

NMR - Solution ^{1}H and ^{13}C spectra were recorded at room temperature on a Varian Gemini 200 NMR spectrometer (200 MHz ^{1}H, 50 MHz ^{13}C). Deuterated water (D_2O) was used as solvent for the dry samples.

Molecular weight determination – ESI (Electrospray ionization) mass spectra were recorded on a Finningan LCQ Deca ion trap mass spectrometer (San José, CA, USA) fitted with an electrospray ion source. Samples were dissolved in H_2O/MeOH (60:40, v/v) with a concentration of 0.4 mg ml^{-1}. Samples were infused into the ESI source at a flow rate of 100 µl min^{-1}. The mass spectrometer spray voltage was set at 4.5 kV, heated capillary temperature

at 275°C, nitrogen (99.5% purity) was used as sheath (0.6 L min^{-1}) and auxiliary (6 L min^{-1}) gas. Mass spectra were recorded in the negative ion mode. The LC-MS system data acquisition and processing were managed by Xcalibur software (version 1.2, Thermo Fisher Scientific). Molecular weights were also determined by gel permeation chromatography in a GPC chromatographer, Agilent 1100, equipped with refractive index detector, using an Ultrahydrogel 120 Waters column. Determinations were conducted at 25°C with a flow rate of 500 µl min^{-1} of filtered and degasified saline solution as solvent. A volume of 20 µl with a concentration of 1 mg ml^{-1} was injected. PEG standards of molecular weight of 106, 440, 628, 960, 1460 and 4290 g mol^{-1} were used for calibration.

DSC - Thermal transitions were evaluated by differential scanning calorimetry (DSC 7 calorimeter, Perkin Elmer) equipped with an external cooling system. Approximately 5 mg of dry oligourethanes samples were heated from -30 to 150°C with a heating rate of 10°C min^{-1}. After first and second scans, the samples were cooled with a cooling rate of 200 °C min^{-1}. Results from the second scan were used to report thermal properties of the oligourethanes such as melting temperature.

2.3. Crosslinking of collagen scaffold and its characterization

2.3.1. Crosslinking of scaffold with oligourethanes

Pericardial matrix scaffold samples were crosslinked with oligomers as follows. [10] Hydrated samples of known mass were immersed in PBS (0.03 M, 0.9% NaCl, pH 7.4, at a ratio (scaffold:PBS, in grams) of 1.0:3.5, then oligourethanes (15w%) were added and the mixture was stirred at 24°C for 2 h under 20 rpm orbital agitation. In a second step, 0.2w% of magnesium oxide was added and the crosslinking reaction extended for 10 h under orbital stirring. Finally, samples were washed with distilled water and PBS containing 0.03 M EDTA at 4°C to remove residual reagents.

2.3.2. Characterization of oligourethanes-pericardial scaffolds

In vitro degradation resistance - The resistance to *in vitro* degradation was studied under enzymatic (collagenase type I, 288 U/mg solid, *Clostridum histolyticum*, pH 7.4, 0.05M Tris•HCl, 0.03w% NaN_3, 0.005 M $CaCl_2$•$2H_2O$) or oxidative (H_2O_2, 20%) conditions. Material of known mass (sample size of 10 x 15 mm) were incubated in 1 ml of the degradation solution under orbital stirring at 37°C for enzymatic test or 25ºC for oxidative test. The collagenase degradation was conducted with a concentration of 1.25 mg enzyme by ml and stopped after 24 h or with a concentration of 0.125 mg enzyme by ml for several days (solution was changed every two days). Finally, the mass loss was calculated gravimetrically while the concentration of free amine groups released from materials after degradation was quantified by the ninhydrin assay. For this, supernatants were heated in ninhydrin solution (1.0w%, citrate buffer pH 5.0) at 95ºC for 20 min and then the absorbance was measured at 567 nm (Uv-Vis spectrophotometer, Beckman Coulter DU 650). The concentration of released amine groups was calculated (using the molar absorptivity coefficient of 1640 ml $mmol^{-1}$ cm^{-1}).

Shrinkage temperature (ST) – The ST was determined by the ASTM D-6076-97 standard method. Material strips (5 x 30 mm) were placed in between two clamps (one fixed and the other mobile) of an Otto Spechs equipment (70435, Stuttgart, Germany) and heated in water from 40 to 90°C at 3°C min^{-1}. The ST was recorded as the temperature when the onset of tissue shrinkage was detected in the mobile clamp.

Blocked matrix-amines - The extent or degree of crosslinking was also evaluated by the change in the free amine content in materials by means of the ninhydrin assay as described

previously, except that the scaffold samples were heated in the ninhydrin solution and the concentration of amines was standardized with the mass of the materials.

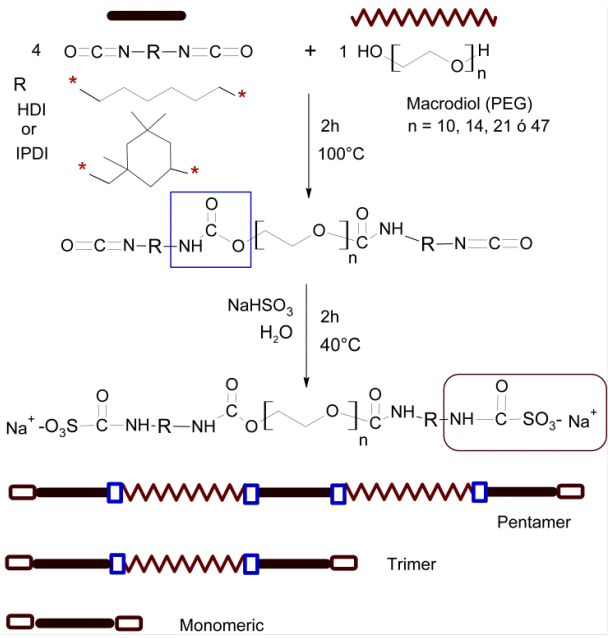
In vitro toxicity assay - Rat dermal fibroblast cells or mouse monocyte macrophage cells (RAW264.7) were routinely grown in DMEM or RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The cells suspended in 200 µl were plated at 50 x 10^3 cells per well in 96-well plates and cultured at 37°C in 5% CO_2 and 100% humidity for 24 h. Cytotoxicity was evaluated using the MTT viability staining assay. Trimmed oligourethane/collagen material samples (small sections, around to 3 x 3 mm) were sterilized by UV irradiation for 3 h and afterward incubated in a sealed tube at 37°C for 48 h in non-supplemented culture medium (20 mg material by ml medium). The supernatants (extracts) were then supplemented with 10% FBS and added either directly (100%) or after dilution (50%, 25% with fresh supplemented medium) onto growing cells (for 24 h).[29] After culture for 24 h more, 10 µL of MTT solution was added to each well, and the cells were maintained under these conditions for 3 h. Finally, the medium was decanted, cells were washed and the blue formazan crystals were dissolved in 2-propanol, and optical density of the supernatants was measured at 540 nm. The absorbance of reduced MTT by cells growing in standard supplemented medium (without extractable product) represented the 100% of cell viability. Moreover, cell viability after culturing in the presence of medium with extracts obtained from uncrosslinked-collagen scaffold (free of oligourethanes) was assessed for comparison purposes.

2.3.3. Statistical analysis

Data sets were compared using one-way analysis of variance (ANOVA). The Sidak-Holm Test was used for the comparison between data groups. The results were considered significant at *P*-values less than 0.05 and presented as mean ± standard deviation (SD).

3. Results and Discussion

PEG of different lengths was reacted with aliphatic diisocyanates to form oligourethane structures. The isocyanate-ended products were blocked with sodium bisulfite [25-26] to yield sodium-carbamoylsulfonate-ended species (Scheme 1). Subsequently blocked products were used to crosslink collagen scaffolds. PEG was chosen for oligourethane synthesis as it is a biocompatible water-soluble polymer, used in the preparation of a variety of biomaterials, while aliphatic diisocyanates HDI and IPDI were preferred over the aromatic ones due to their low toxicity and reactivity that reduces undesirable side reactions. [17]



Scheme 1

3.1. Chemical structure of the oligourethane crosslinkers

FTIR spectra showed characteristic bands of secondary amine groups (3331 cm^{-1}), carbonyl (C=O) groups (1675 cm^{-1}), and the bending mode of N-H bond and stretching mode of C-N bond (1520 cm^{-1}). In Figure 1, FTIR spectra for PEG, H-10 and I-10 are showed; for the other

blocked oligomers, FTIR spectra were similar (Figure S1). The isocyanate (NCO) and urethane (NHCOO) groups of the prepolymer and blocked oligourethane were also detected by FTIR (Figure S2). In the spectrum of the blocked oligourethane (Figure S2b), there was no absorption in the 2263 cm^{-1} range, which indicated that the -NCO groups of oligourethane were completely blocked by sodium bisulfite. The band at 1206-1209 cm^{-1} indicated S=O vibration in the spectrum of the oligourethane blocked with sodium bisulfite (Figure 1).

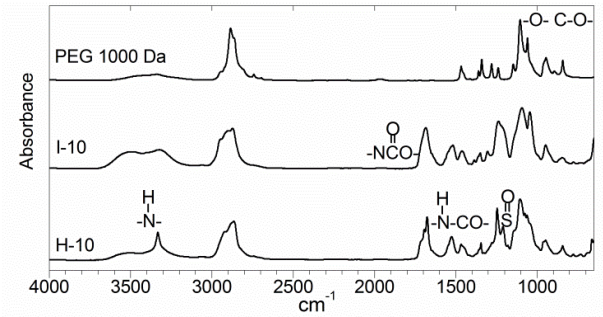


Figure 1

^{1}H NMR spectra revealed the formation of sodium-carbamoylsulfonate-ended-species (Figure 2a and 2b for H-10 and I-10, respectively). Methylene protons of PEG segment, corresponding to PEG backbone and those adjacent to oxygen on the urethane groups were observed (labeled as peaks *a* and *b*, respectively, in Figure 2a and 2b). Methylene protons from HDI segments adjacent to nitrogen on the urethane groups were labeled as peak *c*, while those protons in the alkyl chain between methylene attached to urethane and methylene linked to carbamoylsulfonate were labeled as peaks *d* and *e* (Figure 2a). IPDI is an asymmetric cycloaliphatic diisocyanate that contains primary (NCO bonded to a methylene group) and secondary (NCO directly bonded to the cyclohexyl) isocyanate groups. It exists as two isomers, 72/28 cis/trans. [30-32] Methyl on cyclohexyl ring from the IPDI segments were labeled as peaks *d* and *e*, while $–CH_2–$ and –CH– on cyclohexyl ring were labeled as peaks *f* and *g* (Figure 2b). The proton signals observed in other products were similar to those reported here as representative examples.

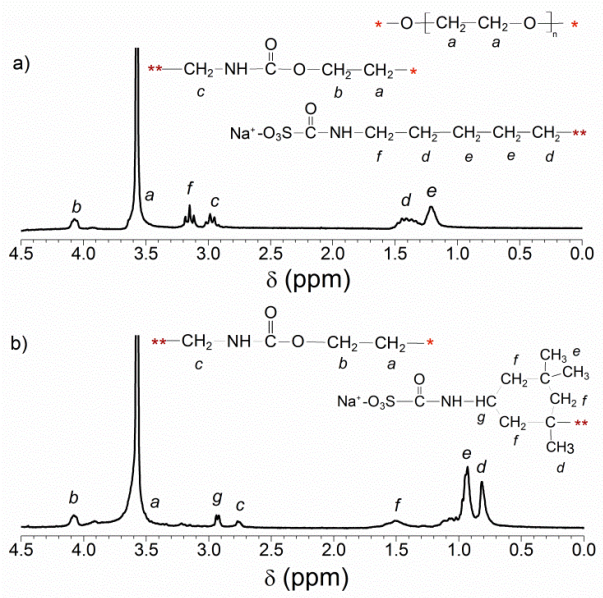


Figure 2

^{13}C NMR spectra revealed two signals in the region for carbonyl groups attributed to two carbonyls in two different chemical environments, i.e., carbonyls in the urethane and carbamoylsulfonate groups (Figure 3). For HDI/oligourethanes, two bands appeared at 157.8 and 165.1 ppm that can be assigned to urethane/C=O and carbamoylsulfonate/C=O groups respectively (Figure 3a). The additional carbonyl signals in Figure 3b may be associated to carbonyls that were formed in the different isocyanate groups in IPDI namely primary and secondary. [30-31] Two bands appeared at 157 and 158.5 ppm can be assigned to secondary and primary urethane/C=O, while bands appeared at 164.1 and 165.6 ppm can be assigned to secondary and primary carbamoylsulfonate/C=O respectively. The shoulders in peaks can also be assigned to cis/trans isomers. [32]

Both FTIR and NMR analyses confirmed the effective blocking of the products obtained from the reaction of PEGs with two aliphatic diisocyanates (symmetric or asymmetric) followed by reaction with sodium bisulfite. In order to confirm the presence of sodium-carbamoylsulfonate ended-species including monomeric diisocyanate and oligourethane structures, the ESI-MS analysis was conducted.

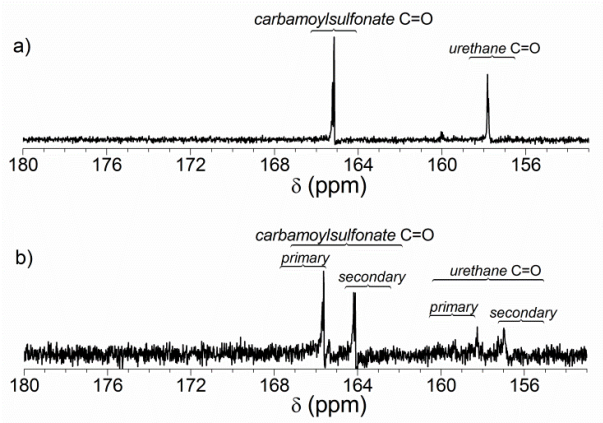


Figure 3

3.2. Molecular weight of the protected-isocyanate oligourethanes

Two examples of representative ESI-MS spectra for water-soluble products synthesized from HDI and IPDI are shown in Figure 4a and 4b, respectively. ESI-MS spectra confirmed the presence of sodium-carbamoylsulfonate ended-monomeric diisocyanate structures in the oligourethane solution (Figure 4a and 4b for H-6 and I-6, respectively, and Figure S3 for others). The two more abundant distributions observed in each spectrum were associated to polydispersed chains with a similar oligourethane structure with one and two charges. The main oligomer structure corresponded to a blocked trimer structure, i.e., isocyanate-PEG-isocyanate (I-PEG-I) chains protected with sodium-carbamoylsulfonate groups. In addition, smaller distributions observed in the ESI-MS spectra revealed some chain extension reaction. Only one additional distribution was observed in IPDI-based product (Figure 4b), while three additional distributions were observed in the HDI-derived product (Figure 4a). These less intense distributions corresponded to blocked-oligourethane substances with pentamer (I-PEG-I-PEG-I) and heptamer (I-PEG-I-PEG-I-PEG-I) structures. The structural and conformational differences between IPDI and HDI have an effect on the side reactions in the urethane oligomer formation. While HDI is a symmetric, linear and flexible molecule with two identical isocyanate groups, IPDI is asymmetric with nonequivalent isocyanate groups of different reactivity.

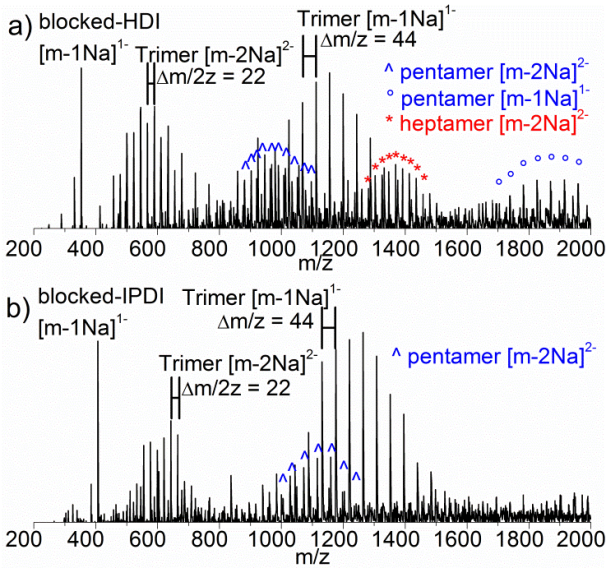


Figure 4

The molecular weight distributions were consistent with the ones reported for the reaction of methylene biphenyl diisocyanate, toluene diisocyanate or IPDI and polyether diols according with a polyaddition. [33-34] The distance between the peaks within each distribution showed the exact mass of the PEG monomeric repeating unit. Table 2 and Table 3 compare the theoretical and calculated masses for the main peak of the single-charged trimer (Table 2) and pentamer or heptamer (Table 3) species. The molecular masses detected by ESI-MS were in agreement with the theoretically expected molecular masses for a sodium carbamoylsulfonate terminated-urethane oligomer (Table 2 and 3).

Table 2. **Molecular mass of the more abundant single-charged sodium carbamoylsulfonate terminated-products**

	Mass of the starting materials (g mol^{-1})			Mass of the blocked products (g mol^{-1})			
Type	PEG[a]	I[b]	$NaHSO_3$	t-I[c]	e-I[c]	t-oligourethane[d]	e-oligourethane[d]
H-6	634.3					1178.8	1178.6
H-10	942.5	168.2		376.32	376.19	1487.0	1486.8
H-20	2089.1					2633.5	2634.4
I-4	458.2		104.06			1110.9	1110.5
I-6	634.3	222.3		430.42	430.23	1287.0	1287.3
I-10	942.5					1595.2	1595.8

[a)] Mass of the macrodiols detected by ESI-MS spectra; [b)] Mass of the diisocyanates. Theoretical (t) and experimental (e) molecular mass of the blocked products: [c)] monomeric diisocyanate and [d)] the more intense peak of the oligourethane with trimer structure.

Table 3. **Molecular mass (g mol^{-1}) of the sodium-carbamoylsulfonate-ended-oligourethane with pentamer and heptamer structures**

Type	$M_{t\text{-}pent}$ [a]	$M_{e\text{-}pent}$ [a]	$M_{t\text{-}hept}$ [b]	$M_{e\text{-}hept}$ [b]
H-6	1981.4	1981.9	2783.9	2784.2
H-10	2597.8	2598.0	3708.5	3709.3
H-20	4890.1	4889.5	7148.1	7147.6
I-4	1791.4	1791.0	2471.9	-[c]
I-6	2143.7	2145.0	3000.3	-[c]
I-10	2760.1	2760.7	3924.9	-[c]

Theoretical (t) and experimental (e) molecular mass of oligourethane with [a)] pentamer and [b)] heptamer structure: experimental molecular mass in more intense peaks; [c)] no detected.

Considering this, it can be assumed that the formation of the oligourethane was achieved by a complete conversion of OH groups into urethane groups, producing only NCO-terminated molecules. Thus, these bifunctional species can act effectively as crosslinker for biomaterials bearing amines. Blocked species present in aqueous solutions exhibited different molar mass distributions given by both molecular distributions of starting PEG diol and extended oligomers, plus blocked monomeric diisocyanate. Therefore, the combined distributions of molecular weight will allow the incorporation of the oligourethane crosslinker into the collagen scaffold in different manner due to their diffusivity according to their molecular masses. [35] GPC chromatograms confirmed the different distributions of the blocked products (H-6, H-10 and H-20) in aqueous solution (Figure 5). The peak at 8.4 ml eluted volume corresponded to the species of protected-monomeric diisocyanate. The other peaks at lower elution volumes corresponded to blocked-oligourethane species with several structures as detected by ESI-MS.

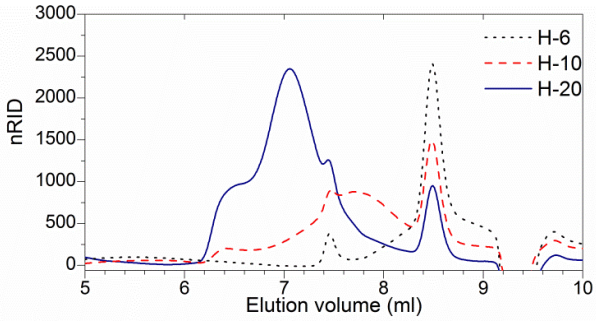


Figure 5

The choice of two aliphatic diisocyanates that differ in structure, i.e., one symmetric (HDI) and one asymmetric (IPDI), can have an effect on the tendency to crystalize of the oligourethanes and finally on properties of oligourethane-collagen scaffolds. The blocked oligourethane H-10 showed cold crystallization, associated to fast crystallization of the linear and flexible PEG segments and melting at 31°C (Figure 6a). Product H-20 also crystallized, and its melting temperature was observed at 45.4°C (Figure 6b). Melting temperature of the PEG chains in the oligourethane derived from HDI increased with the molecular weight of the PEG. The increase of the melting point was also reported for alternating polyurethanes based on L-lysine ethyl ester diisocyanate or HDI and PEG of different molecular weight [36] and attributed to an increase in the size of the crystallites, because of the increased chain length of PEG. [37] The protected oligourethanes have two intrinsic advantages, hydrophilicity and chain flexibility, dictated by the poly(ethylene oxide) chains that may facilitate their penetration through the materials in aqueous medium. The flexibility of the linear macromolecules of the oligourethane was inferred from cold crystallization observed in H-10 and H-20 (Figure 6), which is generally observed in flexible linear macromolecules when a glassy sample is heated after vitrification by quenching from the melt. [38] For oligourethanes synthesized from IPDI no melting endotherms were observed, which can be related to the steric hindrance of the asymmetric groups of IPDI that reduces the tendency for phase separation. It has been reported that polyurethanes with amorphous soft segments degrade more rapidly than those with semicrystalline soft segments. [39-40] This observation has been related to a hindered water absorption due to higher crystallinity. [41]

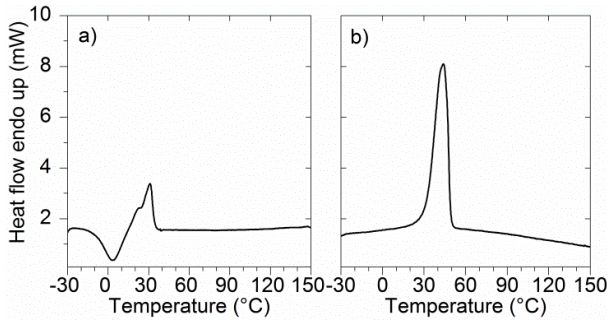


Figure 6

3.3. Effects of the chemical structure and Mw of oligourethanes on the crosslinking degree of collagen scaffolds

A relationship between the crosslinking extent of the oligourethane-collagen material and the Mw of the oligourethanes was observed (Figure 7). The crosslinking of collagen scaffold with H-20 or I-10, the oligourethanes of higher Mw, produced both the lowest content of blocked collagen-amines and the lowest shrinkage temperature. The tendency to increase the crosslinking degree with the decrease of the Mw of the oligomer was found for both the HDI and the IPDI oligourethanes. The process of collagen crosslinking with oligourethane is the result of the reaction between two reactive sites in the protein (primary amines) and deblocked isocyanate groups after increasing the pH. [10,28] The crosslinking extent of the collagen scaffold can be limited by diffusion phenomena related to the Mw of the oligourethane molecules. The pH, time and volume are process variables that regulate the crosslinking degree in the preparation of oligourethane-collagen material. [10] In this study, the macromolecular nature of the oligourethane is reported as an additional variable to prepare a library of hybrid scaffolds with specific controlled crosslinking degree. Swelling and mechanical behavior are two important aspects for a tissue engineering scaffold. It has been reported that when compared with glutaraldehyde or EDC carbodiimide crosslinker, dehydration induced by crosslinking of acellular pericardial scaffold with oligourethane (H-10) is not so severe. [10] Tensile viscoelastic properties of acellular pericardial scaffold are modified after its crosslinking with oligourethane (H-10). However, to prove if the swelling or mechanical behavior of oligourethane-collagen scaffolds are influenced by the molecular weight of oligourethane additional studies are required.

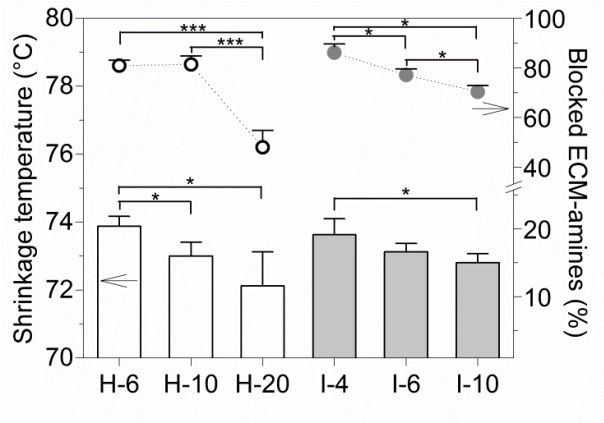


Figure 7

3.4. Effects of the chemical composition on the *in vitro* degradation of oligourethane-collagen scaffolds

After collagenase degradation, the remaining mass in all oligourethane-collagen materials was above 90% while the concentration of soluble fragments of collagen released to the solution (supernatant) after cleaving peptide bonds from material was very small (Figure 8). Whereas the lower crosslinking degree was found in H-20 treated scaffold (Figure 7), the lower resistance to collagenase (360 U) was detected in the same material after incubation for 24 h (Figure 8).

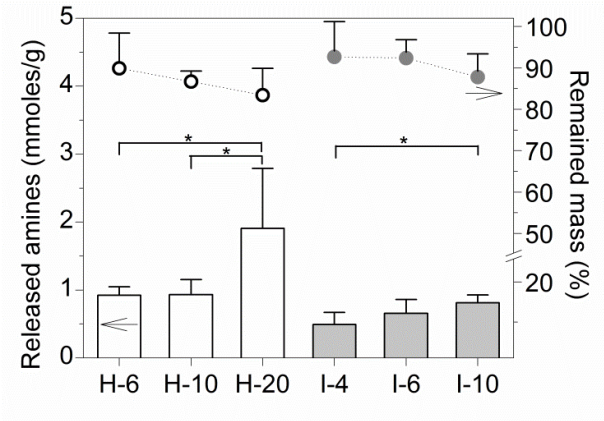


Figure 8

The tendency to decrease in the resistance to enzymatic degradation with the increase of the Mw was found for both the HDI and the IPDI oligourethanes. The crosslinked scaffolds allowed for smaller interstitial spaces, which in turn limited enzyme infiltration.[42] So, the initial degradation could be limited to superficial damage. Moreover, the macromolecular chains of oligourethane can protect peptide bonds of the collagen in all their hierarchical structural levels by increased coating or masking of the α chain or triple helical structure.[43] However, enzymatic degradation and oxidative degradation revealed a strong dependence on the oligourethanes structure. The crosslinking of collagen scaffold with oligourethane derived from IPDI led to a higher susceptibility to *in vitro* degradation (enzymatic or oxidative) than the ones crosslinked with HDI/oligourethane (Figure 9). The different degradation behavior between I-10/scaffold and H-10/scaffold (Figure 9) can be related to the hydrophilicity difference between H-10 and I-10 oligourethanes associated to a tendency for phase separation (and enhanced crystallinity) in H-10 respect to I-10. However, more studies must be carried out in order to evaluate if the biodegradation of the oligourethane-collagen scaffolds is dependent on the ability of the oligourethanes to crystallize.

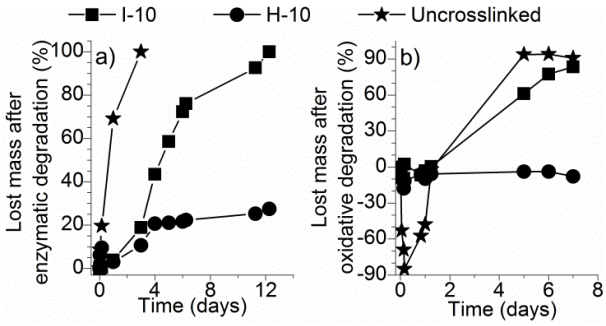


Figure 9

3.5. Effects of the chemical composition on the cytotoxicity of leachable products from oligourethane-collagen scaffolds

Macrophages are key players in the release of enzymes, cytokines and growth factor that act as signals to induce migration, proliferation or differentiation for other cell types such as

fibroblasts. [8,44] Moreover, macrophages mediate the healing responses to implanted biomaterials, fundamentally by two outcomes: scar formation (M1 pathway) or regeneration (M2 pathway). [5,44] The potential release of toxic compounds from oligourethane-collagen scaffolds was studied by means of quantification of the viability of fibroblasts and macrophages cultured with extracts obtained at 37°C. The extracted compounds from materials crosslinked with oligourethanes differing in their chemical structure showed a higher cytotoxic potential for fibroblasts in H-10 than in I-10 crosslinkers (Figure 10). This effect was ameliorated when diluted conditioned media was used in all groups. It is possible that the leachable products from scaffolds crosslinked with H-10 were compounds more reactive in comparison to I-10 influenced by differences in the chemical structure and the NCO group reactivity between HDI and IPDI. Therefore, the cytocompatibility of the oligourethane-collagen materials could be improved by means of the crosslinking chemistry related to diisocyanate structure. Diisocyanates that generate non-toxic degradation products have been explored in the synthesis of suitable biomaterials for tissue engineering. For instance, ethyl lysine diisocyanate-crosslinked gelatin hydrogels showed favorable interactions with fibroblasts as well as human mesenchymal stem cells in both cells cultured with material extracts and cells seeded on materials. [13] Thus, it is necessary to test if cells seeded onto oligourethane-collagen scaffolds are able to proliferate and it would be interesting to investigate if leachable/degradation products have an influence on the macrophage pathways.

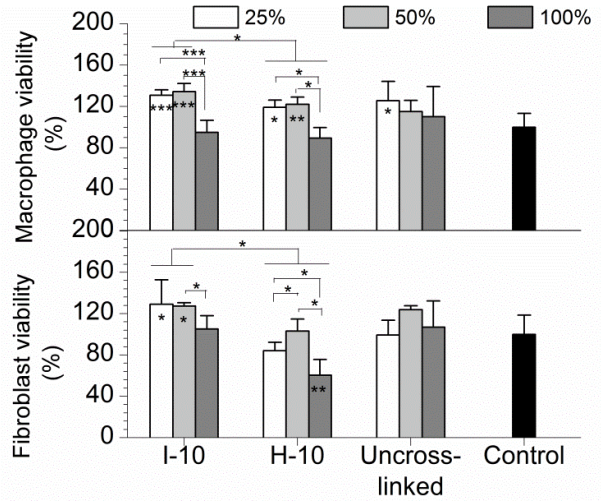


Figure 10

Finally, the reported battery of protected-oligourethanes had the ability to crosslink proteinaceous material, as demonstrated by their application in the crosslinking of pericardial matrix scaffolds. The extrapolation of the oligourethane as crosslinker for other protein-based biomaterials is possible, and studies related to the biological response to natural scaffold modified with these oligourethanes are required. Based on the results reported here, this approach is promising in the fabrication of collagen scaffolds with properties tailored by the crosslinking process or the development of multicomponent hydrogels by isocyanate chemistry.

4. Conclusions

Water-soluble oligourethanes were produced after macrodiisocyanates synthesized from PEG and aliphatic diisocyanates (I) were blocked with sodium bisulfite. The oligourethanes with sodium carbamoylsulfonate end groups were composed of trimeric (I-PEG-I) and pentameric (I-PEG-I-PEG-I) structures and blocked-monomeric diisocyanate. The library of oligourethanes differing in the structure of diisocyanate (IPDI or HDI) and Mw of PEG (600 to 2000 g mol^{-1}) were able to crosslink a decellularized pericardium, as a model collagen scaffold. The degree of crosslinking and subsequently the resistance to *in vitro* degradation of

the crosslinked collagen-rich scaffolds were dependent on the Mw of the urethane oligomers. The degree of crosslinking was not significantly affected by the structure of diisocyanate, although the susceptibility to *in vitro* degradation and the cytotoxic potential of the soluble product appeared to be dependent on diisocyanate chemistry.

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Scheme Caption:

Scheme 1. **(**Up) Reaction scheme for oligourethanes derived from hexamethylene diisocyanate (HDI) or isophorone diisocyanate (IPDI). * nitrogen of NCO groups. **(**Below) Schematic representation of the structure of the water-soluble blocked crosslinker.

Figure captions:

Figure 1. ATR-FTIR spectra for representative PEG and blocked-urethane oligomers (H-10 and I-10).

Figure 2. ^{1}H NMR spectra for representative blocked-urethane oligomers a) H-10 and b) I-10.

Figure 3. Carbonyl signals in ^{13}C NMR spectra for representative blocked-urethane oligomers a) H-10 and b) I-10.

Figure 4. Electrospray mass spectra of representative blocked-urethane oligomers a) H-6 and b) I-6.

Figure 5. GPC chromatograms of blocked-urethane oligomers synthesized from hexamethylene diisocyanate.

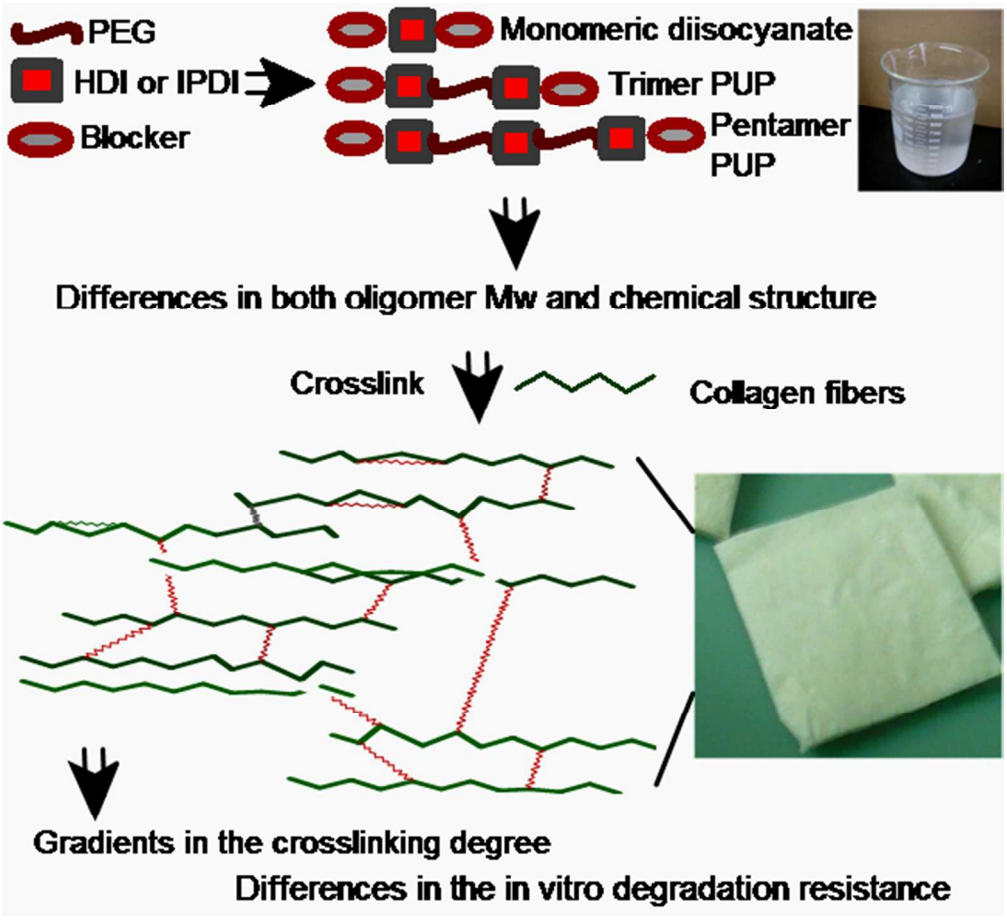
Figure 6. DSC thermograms for blocked-urethane oligomers a) H-10 and b) H-20.

Figure 7. Shrinkage temperature (bars) and blocked-amines (dots) for pericardial scaffolds treated with oligourethanes; mean ± SD; n=4; ANOVA: * $P<0.05$, *** $P<0.001$ for all marked groups.

Figure 8. Released amines (bars) and remaining mass (dots) after collagenase degradation (1.25 mg ml^{-1} enzyme, 24 h) for pericardial scaffolds treated with oligourethanes; mean ± SD; n=4; ANOVA: * $P<0.05$ for all marked groups.

Figure 9. In vitro collagenase (0.125 mg ml^{-1}) and oxidative degradation profiles for pericardial scaffolds uncrosslinked or crosslinked (H-10 or I-10); duplicate.

Figure 10. Viability assessed by MTT for (a) fibroblasts or (b) macrophages cultured in the presence of three different concentration of culture media with extracts of scaffolds uncrosslinked or crosslinked (H-10 or I-10); n=4; ANOVA: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ for all marked groups or for groups compared to control (marks inside column). Control: Viability of cells growing in standard supplemented medium (without extracts).



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