Photo-crosslinked poly(D,L-lactide)-based networks. Structural characterization by HR-MAS NMR spectroscopy and hydrolytic degradation behavior

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ABSTRACT

To date, biodegradable networks and particularly their kinetic chain lengths have been characterized by analysis of their degradation products in solution. We characterize the network itself, by NMR analysis in the solvent-swollen state under magic angle spinning conditions. The networks were prepared by photo-initiated crosslinking of poly(D,L-lactide)-dimethacrylate macromers (5 kg/mol) in the presence of an unreactive diluent. Using diffusion filtering and 2D correlation spectroscopy techniques, all network components are identified. By quantification of network-bound photo-initiator fragments, an average kinetic chain length of 9±2 methacrylate units is determined. The PDLLA macromer solution was also used with a dye, to prepare computer-designed structures by stereolithography. For these networks structures, the average kinetic chain length is 24±4 methacrylate units. In all cases the calculated molecular weights of the polymethacrylate chains after degradation are maximally 8.8 kg/mol, which is far below the threshold for renal clearance.

Upon incubation in phosphate buffered saline at 37 °C, the networks show a similar mass loss profile in time as linear high-molecular weight PDLLA (HMW PDLLA). The mechanical properties are preserved longer for the PDLLA networks than for HMW PDLLA. The initial tensile strength of 47±2 MPa does not decrease significantly for the first 15 weeks, while HMW PDLLA lost 85±5 % of its strength within 5 weeks. The physical properties, kinetic chain length and degradation profile of these photo-crosslinked PDLLA networks make them most suited materials for orthopedic applications and use in (bone) tissue engineering.

KEYWORDS polymer network, nmr, magic angle spinning, hr-mas, hydrolytic degradation, biodegradable.

Introduction

Photo-initiated networks that degrade in the body are of great interest for biomedical applications. They can serve as surgical implants,[1] glues,[2] drug delivery devices[3] or as scaffolding materials for tissue engineering.[4, 5] The ease with which they can be prepared and their good mechanical performance enhance their utility in these applications. By altering the chemistry, material properties such as mechanical properties, hydrophobicity, cell-material interactions and degradation kinetics can be tailored.[6] Furthermore, photo-initiated networks exhibit good shape stability due to their crosslinked nature, even at temperatures higher than the polymer's glass transition temperature.[7, 8] Photo-initiation has several advantages over other methods of network formation: it is rapid, and temporally and spatially controlled. In cell encapsulation[9] and in the preparation of injectable hydrogels that can be cured transdermally, mild reaction conditions are particularly important.[4, 10] Biodegradable networks have often been prepared from hydrolytically degradable oligomers composed of monomers such as lactide, caprolactone, and trimethylene carbonate, after end-functionalization with methacrylate,[7] acrylate[11] or fumarate groups.[12] Upon light irradiation in the presence of a photo-initiator, the end-groups polymerize via an addition-type reaction to form polyvalent crosslinks, and a network is formed.

In vivo, polymers and their networks usually degrade by a combination of hydrolytic and oxidative degradation, with or without mediation by enzymes. Hydrolysis reactions lead to scission of polymer (network) chains, and take place either through a surface erosion[13] or bulk degradation mechanism[14]. The kinetics of hydrolytic degradation of such networks is governed mainly by the type of degradable bond and by the hydrophilicity of the network.[13] For example, anhydride bonds are hydrolyzed much faster than ester bonds[15], and the carbonate bonds in poly(trimethylene carbonate) are hydrolyzed particularly slow.[16] On the other hand, the amount of water that can be taken up by the network strongly influences the rate of chain scission. Degradation times can vary from several days for hydrogels consisting of poly(ethylene glycol) chains extended with lactide blocks and methacrylate groups,[11] to up to more than one year for hydrophobic ε-caprolactone[17] and networks thereof. Furthermore,

components present in the network that attract water, such as solvents or residual monomer, may significantly speed up the hydrolytic degradation,[8, 18] this is also the case for linear polymers.[19]

After complete hydrolysis, the degradation products of biodegradable networks are usually water-soluble compounds derived from the polymer network main chains, initiators used in the oligomer synthesis, and polyaddition chains formed in the photo-crosslinking process. The length and character of these chains determines if they can be removed from the body by renal clearance. Water-soluble polymers with molecular weights up to 50 kg/mol are cleared by the kidneys within a few days. Above 50 kg/mol, liver clearance via the uptake of Kupffer cells predominates.[20] A maximum accumulation in the circulatory system has been observed for polymers which have a molecular weight over 200 kg/mol, regardless of polymer type.[21] For this reason, the length of the kinetic chains in biodegradable networks has been the subject of a number of studies.[22-25] It can vary significantly depending on the photo-crosslinking conditions; however, exact chain lengths have been difficult to quantify. For example, kinetic chain lengths of poly(methacrylic acid) chains from sebacic acid-dimethacrylate based networks were studied using mass spectrometry[23] in one study and using gel permeation chromatography in another study.[22] The networks were prepared under similar conditions, but the outcomes were very different: average chain length values reported were 10-40 or 1000-2000 monomer units, respectively.

In this work we present the characterization of photo-crosslinked poly(D,L-lactide) (PDLLA), and its hydrolytic degradation profile. Due to their good mechanical properties, these networks have great potential for orthopedic and bone tissue engineering applications. Furthermore, they can be prepared in almost any desired shape using stereolithography.[26] In this technique, a computer-controlled laser beam or a digital light projector is used to locally solidify a liquid resin through photo-initiated polymerization. In combination with a computer-driven building stage, a solid, three-dimensional computer-designed object can be constructed in a layer-by-layer fashion.

To date, such biodegradable networks and particularly their kinetic chain lengths have been characterized by analysis of their degradation products in solution. We characterize the molecular structure of networks photo-crosslinked from PDLLA-dimethacrylate precursors using sophisticated

nuclear magnetic resonance spectroscopy techniques. This enables determination of the kinetic chain length without the need for degradation. Furthermore, we present the development of network properties, water uptake, mass loss and mechanical properties in time, during degradation at 37 °C in phosphate buffered saline.

Experimental

Hydroxyl-terminated oligomers were synthesized by ring-opening polymerization (130 °C, 40 h under an argon atmosphere) of D,L-lactide (DLLA, Purac Biochem) using 1,6-hexanediol (Sigma-Aldrich) as initiator and stannous octoate (Sigma-Aldrich) as catalyst. The monomer-to-initiator ratio was adjusted to yield oligomers with a molecular weight of 5 kg/mol. The hydroxyl-terminated oligomers were reacted with methacrylic anhydride (Sigma-Aldrich) in the presence of triethyl amine (Sigma-Aldrich) (both in a 20 mol % excess) in dried dichloromethane (Biosolve) for 5 days to yield methacrylate end-functionalized lactide macromers. The macromers were purified by precipitation from isopropanol (Biosolve), followed by washing with water and freeze-drying.

With the macromers, a liquid photo-crosslinkable resin was prepared, consisting of 58 wt% PDLLA-dimethacrylate, 40 wt% dry N-methylpyrrolidone (NMP, Fluka) as an unreactive diluent, 2 wt% ethyl-2,4,6-trimethylbenzoylphenylphosphinate (Lucirin TPO-L photo-initiator as a gift from BASF), 0.2 wt% α-tocopherol inhibitor (Fluka) to prevent preliminary crosslinking and 0.15 wt% Orasol Orange dye (gift from BASF). Using this resin and a commercial stereolithography apparatus (Envisiontec Perfactory Mini Multilens SLA), computer-designed porous structures with a gyroid design were fabricated as described elsewhere.[27] The stereolithography apparatus was also used to prepare films by projecting a 75x60 mm^2 rectangle of blue light (intensity 10 mW/cm^2) onto a 0.64 mm thick layer of resin for 5 min. In this case, a dye was not required in the resin. The solvent-swollen crosslinked films were washed in acetone (Biosolve) and postcured by irradiating with 365 nm UV-light for 15 min (Ultralum crosslinking cabinet, intensity 3–4 mW/cm^2) under a nitrogen atmosphere. The diluent and unreacted photo-initiator were extracted from the films with acetone. The films were first dried at

ambient conditions, after which the presence of a small acetone solvent residue allowed the effortless punching of disks (Ø 5 mm) and strips (4.8x60 mm) from the plasticized 0.5 mm thick films. The disks and strips were finally dried at 90 °C for 2 days under a nitrogen flow. Glass transition temperatures were determined using a Perkin-Elmer Pyris 1 differential scanning calorimeter (DSC) at a heating rate of 10 °C/min. The glass transition temperature was taken as the midpoint of the heat capacity change in the second heating run, after quenching (300 °C/min).

PDLLA-dimethacrylate macromer, photo-initiator Lucirin TPO-L and degradation product of the PDLLA network were analyzed in solution (DMSO-d_6) by NMR spectroscopy, using a high-resolution solution probe (triple-nucleus TXI probe with z-gradient) interfaced with a Bruker Avance II 600 spectrometer operating at 600.34 MHz (^{1}H) or 150.2 MHz (^{13}C), and equipped with a Great 3/10 gradient amplifier.

Photo-crosslinked PDLLA was analyzed in the solvent-swollen state using a high resolution magic angle spinning (HR-MAS) probe equipped with gradient coil along the rotor axis. The swollen networks were measured in 50 μL KelF rotors, spinning at 6 kHz. Regular 1D spectra were recorded, as well as 2D correlation spectra (COSY) and nuclear Overhauser effect spectra (NOESY) of the network material.

Pulsed-field gradient stimulated echo (PFGSE, or diffusion-filtered) 1D ^{1}H-NMR spectra were obtained using the bipolar stimulated echo sequence. Typically, field gradient strengths varying from 5 to 30 % of the maximum field strength, 128 averages per increment step, and 100 ms diffusion times were employed. Standard pulse sequences for homonuclear (HH-COSY and HH-NOESY), heteronuclear (HMQC and HMBC) and PFGSE experiments were taken from the Bruker library.

To investigate polymer (network) degradation behavior, disk-shaped specimens (mass m_0) were placed in glass vials containing 5 mL phosphate buffered saline (PBS, supplemented with 0.02 wt% sodium azide (Sigma-Aldrich)) in a 37 °C water-bath under light agitation. The incubation medium PBS was refreshed every 2 weeks. At each time-point, 3 specimens were taken out, washed in demi-water, blotted and weighed (m_{wet}). Subsequently, the specimens were freeze-dried, weighed again (m_{dry}) and

kept in dry NMP to reach equilibrium swelling (minimum 2 days). After blotting and weighing ($m_{swollen}$), the specimens were extracted with acetone, dried at 90 °C for 2 days under nitrogen flow and weighed again (m_{gel}). Gel contents were calculated as m_{gel}/m_0 and the volume degrees of swelling were calculated using the swollen mass $m_{swollen}$ and the densities of PDLLA (1.25 mg/mL) and NMP (1.03 mg/mL):

$$Q = 1 + \frac{m_{swollen} - m_0}{m_0} \times \frac{\rho_{PDLLA}}{\rho_{NMP}}$$

Tensile test strips were kept at 37 °C as well, in 15 mL centrifuge tubes filled with PBS. Also here, the incubation medium was supplemented with 0.02 wt% sodium azide and refreshed every second week. At each time-point, 4 strips were taken out and subjected to tensile testing in the wet state using a Zwick Z020 universal tensile tester at a rate of 10 mm/min. The grip-to-grip separation was 50 mm and mechanical extensometers were employed.

Of the photo-crosslinked PDLLA network film, 13.70 g was degraded in 0.2 L of 1.0 M NaOH (Merck) solution at 100 °C for 3 weeks, in a Teflon round-bottom flask equipped with a reflux condenser. After neutralizing with 37 wt% HCl solution (Merck), the contents were dialyzed to milliQ-water using a cellulose ester dialysis tubing (Spectra/Por, Spectrum Laboratories) with a molecular weight cut-off of 500 Da to remove lactic acid, hexanediol and salts. After dialysis and freeze-drying, the remaining degradation products were dissolved in DMSO-d_6 (Sigma-Aldrich) for NMR analysis as described above.

Linear, high-molecular weight PDLLA (HMW PDLLA) reference material was obtained from Purac Biochem. Granules were compression molded (140 °C, 250 kN, 3 min) to 0.5 mm thick films. From these films, disks and strips of the same dimensions as mentioned before were punched out while plasticizing the polymer at approximately 45 °C (10 °C below the glass transition temperature). Molecular weights and intrinsic viscosities were determined after compression molding using a Viscotek GPCmax gel permeation chromatography setup with Viscotek 302 Triple Detection Array and $CHCl_3$ as an eluent with a flow of 1 mL/min. Initial molecular weights of the polymer after compression

molding were $\overline{M}_n$ =120±5 kg/mol and $\overline{M}_w$ =289±1 kg/mol, intrinsic viscosity was 2.38±0.06 g/dL. NMR analysis ($CDCl_3$, Varian 300 MHz) indicated the presence of 1.7±0.4 % residual lactide monomer in the polymer material.

Unless mentioned otherwise, results are presented as average values ± standard deviations of triplicate measurements.

Results and Discussion

Preparation of photo-initiated poly(D,L-lactide) networks

Figure 1 shows the ^{1}H-NMR spectrum of the synthesized poly(D,L-lactide)-dimethacrylate macromer that was used to prepare the PDLLA networks, and the assignment of all proton resonances. The number-average degree of polymerization of lactide can be derived from the ratio of the integral values of the peaks corresponding to the lactide methine proton (c) and the protons of the hexane methylene that links to the lactide chain (e). This ratio is 35.1±2 lactic acid units per hexanediol-fragment terminus (a total of 70.2±4 lactic acids units in the oligomer), corresponding to a number-average molecular weight of the macromers of 5.1±0.3 kg/mol. By comparing the integral value of the hexane methylene peak (e) to those of the methacrylate $=CH_2$ proton peaks (k), quantitative functionalization of the macromer termini can be concluded. Furthermore, a trace of isopropyl alcohol (IPA) is observed, resulting from the macromer purification.

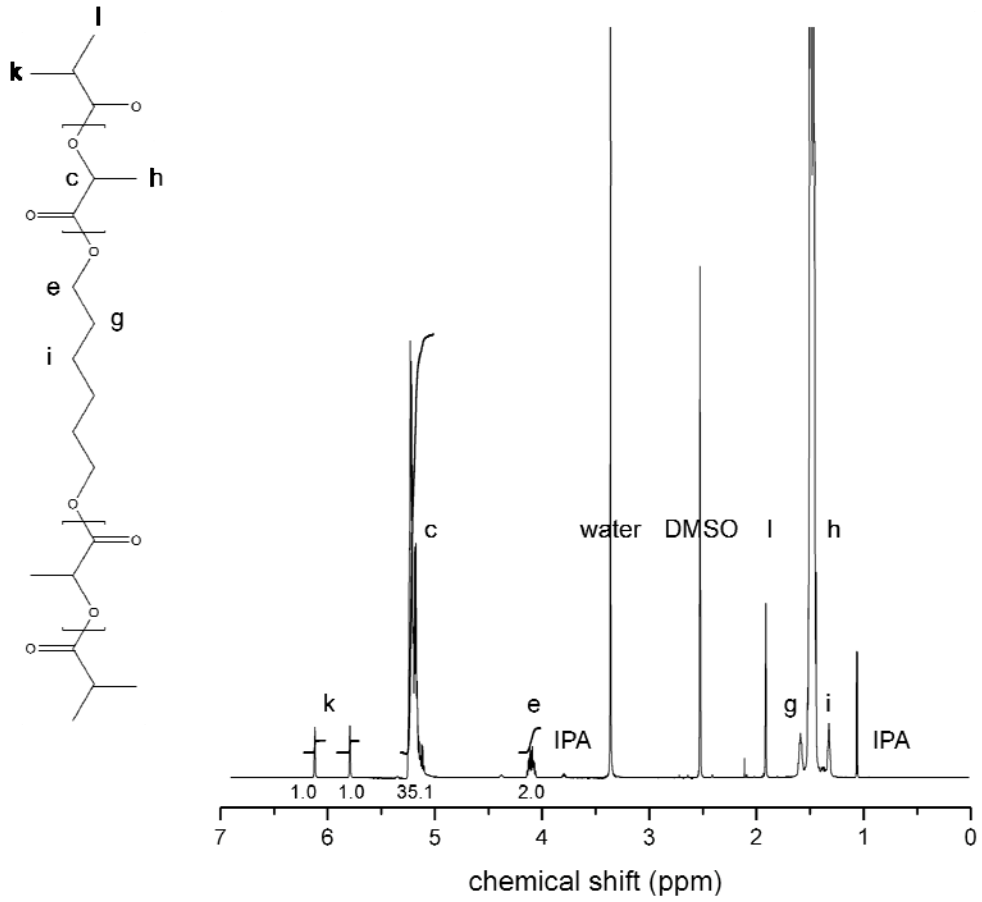

Figure 1. Chemical structure and ^{1}H-NMR spectrum of the PDLLA-dimethacrylate macromer in DMSO-d_6. The labels in the structure and spectrum correspond to those in the network structure depicted in Figure 2. The integrals are normalized with respect to peak e, the integral values in the spectrum reflect the numbers of protons in half of the macromer structure.

The PDLLA macromers were used with an unreactive diluent and photo-initiator (PI) to prepare network films in a stereolithography apparatus. The chemical structure of the photo-initiator that was used, Lucirin TPO-L, and its ^{1}H-NMR spectrum, can be found in the Supporting Information. Upon light irradiation, the photo-initiator produces two different radical species: a phosphinate species (PI_p) and an alkanoate species (PI_a). Both can initiate polymerization of the methacrylate end-groups of the macromers, by which the network is formed. Figure 2 is a representation of the molecular structure of such a photo-crosslinked PDLLA network, in which characteristic protons are labeled for later reference. Note that from each methacrylate unit, a hydrolysable chain consisting of two PDLLA

segments connected by a hexanediol residue originates that is linked to another polymethacrylate chain. Each of the kinetic chains shown in the schematic network structure was initiated by a different radical species, originating from the heterolytic scission of the photo-initiator.

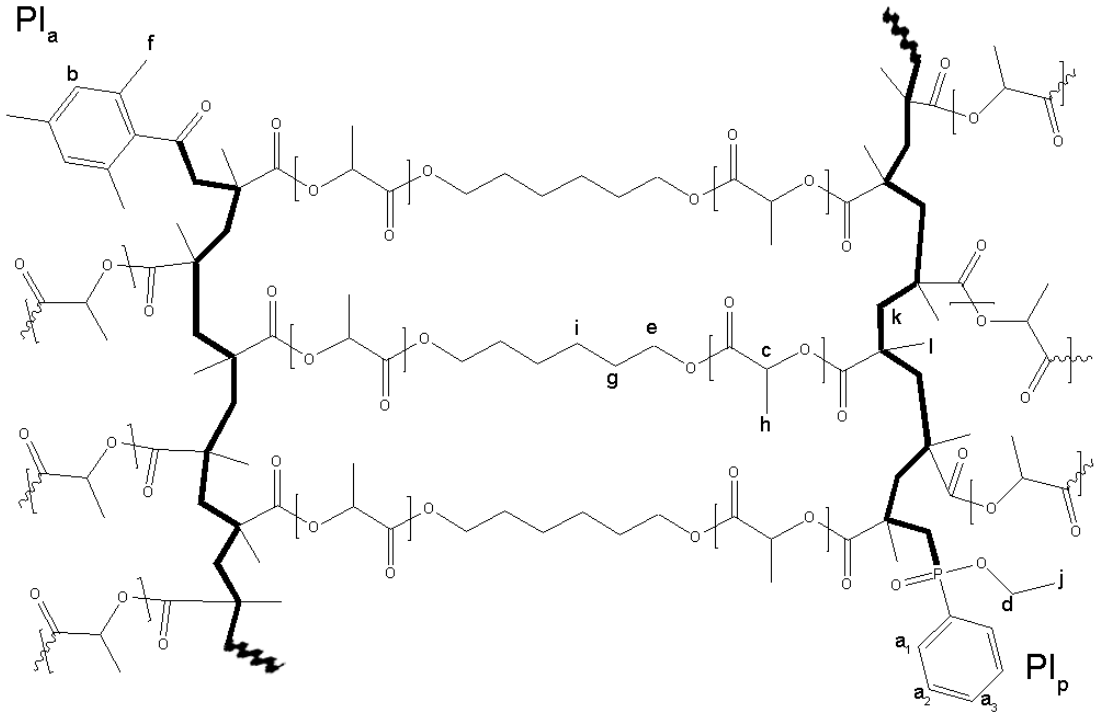

Figure 2. Chemical structure of a photo-crosslinked PDLLA network, in which characteristic protons are labeled for further reference. The polymethacrylate kinetic chains are drawn in bold.

The photo-initiated crosslinking of the PDLLA-dimethacrylate macromers in the liquid resin yields flexible solvent-swollen gels, which after extraction and drying become rigid, glassy networks. The physical properties of these networks are quite similar to those of linear high-molecular weight PDLLA: elastic modulus of 2.6±0.1 GPa, tensile strength of 47±2 MPa and elongation at break of 2.3±0.3 %. The networks show low water uptake of 0.7±0.1 wt% and a glass transition temperature of 58±1 °C. As the tensile strength approaches that of cortical bone (60-100 MPa[28]), these biodegradable networks may be well suited for certain orthopedic applications, for example as fracture fixation devices.[29] Bone tissue engineering scaffolds with various porosities (59-71 %) and pore sizes (0.4-0.7 mm) were prepared

from this material by stereolithography[27], These scaffolds showed compressive moduli (150-360 MPa) and compressive strengths (6-11 MPa) that were comparable to those of cancellous bone (with compressive moduli of 20-500 MPa and compressive strengths of 4-12 MPa). [28]

Network characterization using NMR

To date, biodegradable networks and particularly their kinetic chain lengths have been characterized by analysis of their degradation products.[23, 25] However, mass spectrometry, gel permeation chromatography and NMR spectroscopy of dissolved degradation products have led to divergent results for kinetic chain lengths. We have set out to fully characterize the network itself by solid state NMR spectroscopy on solvent-swollen networks. High resolution ^{1}H-NMR data of the PDLLA network were obtained with an HR-MAS probe. As a result of spinning of the sample at the magic angle[30-33] the signals are sharp, and remarkably high resolution spectra of the networks are obtained (Figure 3). The polymeric material yields broad peaks due its heterogeneous character; there is a distribution of chain lengths and many chiral centers are present. Isolated peaks with characteristic 3-bond spin-spin coupling patterns of a few hertz are also revealed. The 1D and 2D NMR data obtained are complementary, and provide information relevant to understanding the structure of the network: the identification of the main components of the network, the conversion of the chain crosslinking reaction, the ratio of the lactide to hexanediol residue, the presence of solvents, and the relative amount of photo-initiator reaction products. Finally, it allows determination of the number-average kinetic chain length of the network.

First, the network spectra are dominated by the intense peaks around 1.4 and 5.2 ppm (Figure 3, insert) corresponding to the lactide methyl (h) and methine (c) protons, respectively. The dynamic range of the HR-MAS data is very large, and peaks with intensities in the range of a few % of the lactide peaks are still well resolved and can be observed. An important observation that follows from the NMR spectra is that in these post-cured networks almost no unreacted methacrylate double bonds could be detected. While the methacrylate part of unreacted macromonomer shows characteristic peaks (k near 6

ppm, and l at 1.8 ppm) in the ^{1}H-NMR spectrum of Figure 1, peaks k and l cannot be discerned in the NMR spectrum of the swollen (crosslinked) network shown in the insert of Figure 3. At the same time peaks e, g and i can clearly be discerned. In the expanded and filtered spectrum of Figure 3, peaks k and l can only just be distinguished, the ratio of the integral values of peaks l to e is calculated to be less than 1 %. With highly crosslinked glassy networks, complete double bond conversion is rarely reached because of severe restrictions on the mobility of the reacting molecules. Attaining conversions close to 100 % is important for the mechanics and biocompatibility of the resultant network, but has been difficult to achieve.[6] In our networks almost complete double bond conversion (> 99 %) is readily reached, as in the presence of an unreactive diluent the mobility of the PDLLA macromer chains is high.

Further, focusing in on the less intense peaks in the network spectrum, more signals than those of the protons of the lactide chains and the hexanediol residue linkers are observed. To discriminate peaks belonging to the network from those of residual solvents or other impurities, diffusion-filtered pulsed field gradient stimulated echo (PFGSE) experiments were performed. Small, mobile molecules have relatively high diffusion constants and therefore the corresponding peaks decrease faster in intensity than the peaks corresponding to the network material. This becomes clear when comparing the spectra in Figure 3, where the insert shows an unfiltered spectrum, and the larger spectrum is obtained after diffusion-filtering. In the unfiltered spectrum, the water peak (3.4 ppm) is of comparable height to the lactide methine peak (c, 5.1 ppm), whereas in the larger, diffusion-filtered spectrum, the relative intensity of the peak is reduced by approximately a factor 20. The residual solvent peak from partially protonated DMSO (i.e. DMSO-d_5, at 2.51 ppm) clearly loses relative intensity upon increase of the gradient strength. Interestingly, slightly upfield at 2.47 ppm from this solvent peak another peak is observed that corresponds to DMSO-d_5 as well, and probably derives from the solvent molecules that are confined within the network and therefore show slower diffusion than the free solvent itself. Also water shows a second more slowly decreasing signal, most probably corresponding to water molecules that are mixed with DMSO within the network structure (3.2 ppm).

After recording diffusion-filtered spectra with different gradient field strengths and normalization to the lactide peaks of the network, peaks of which the intensity decreased by approximately 50 % were labeled as corresponding to residual solvents and impurities. The remaining peaks could be identified as corresponding to the network. Subsequently, the assignment of the network peaks was confirmed by 1D H spectroscopy, homonuclear 2D HH correlation spectroscopy (COSY) and/or nuclear Overhauser effect spectroscopy (NOESY). The coupling of the lactic acid (CH_3-CH) peaks (h) and (c) is readily confirmed by the COSY spectrum (Figure 4). The COSY spectrum also identifies the proton signals of the hexane linker unit bridging the polylactide chains, connecting the 4.06 ppm peak (e) to the 1.55 ppm peak (g) just downfield from the lactide methyl peak, which consecutively shows a coupling to a peak (i) just upfield from the lactide peak. The length of the PDLLA chain that connects the polymethacrylate backbone and the hexane bridging unit can be calculated from the ratio of the smaller hexane methylene (e) to the lactic acid methine (c) peak in the HR-MAS spectrum of the gel (Figure 3), and is equal to the chain length obtained from the high-resolution NMR spectrum of the macromer in solution (Figure 1), 35±2 lactic acid units.

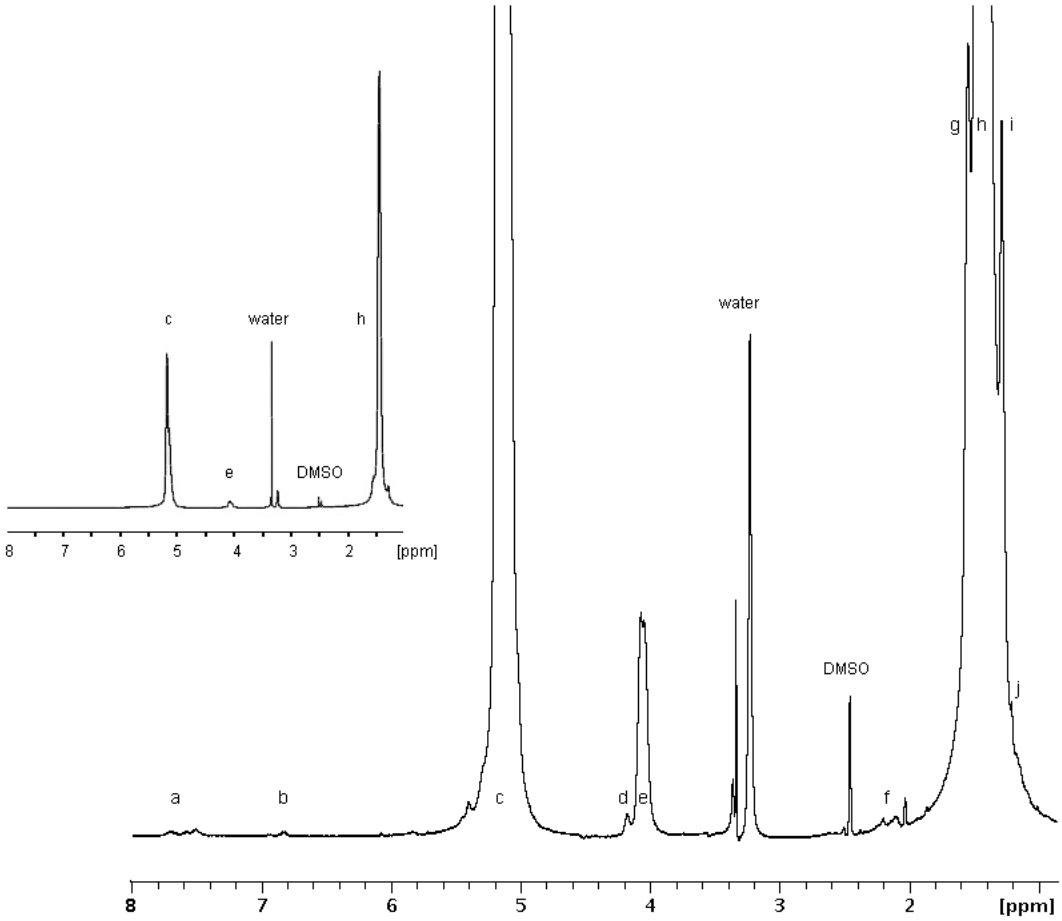

Figure 3. ^{1}H-NMR HR-MAS spectra of a photo-crosslinked PDLLA network swollen in DMSO-d_6. The insert shows the unfiltered spectrum, and the larger expanded spectrum was obtained after diffusion-filtering. The peak labels correspond to those in the network structure depicted in Figure 2.

In Figure 3, the diffusion-filtered spectrum shows small peaks in the aromatic region (6.8-7.8 ppm). These peaks belong to groups that are bound to the network, as can be concluded from the series of PFGSE experiments in which these peaks show the same signal attenuation as the other signals from the network. Comparison with the spectrum of the photo-initiator (Supporting Figure 1) indicates that these probably arise from PI-residues that initiated the chain crosslinking polymerization. Also the small peaks at 1.3, 2.2 and 4.17 ppm can be attributed to the photo-initiator fragments that have initiated the polymerization of the methacrylate end-groups and therefore define the starting points of the polymethacrylate chains (Figure 2). The peaks at 6.8 (b, singlet) and 2.2 ppm (f, singlet) are from the

initiating alkanoate species (PI_a), corresponding to the aromatic and methyl protons, respectively. Their assignment was further confirmed by a NOESY cross-peak (Supporting Figure 2). The other three broad signals more downfield in the aromatic region (a) correspond to the phenyl ring protons of the phosphinate species (PI_p). The ethoxy group of the PI_p unit shows peaks at 4.17 (d) and 1.3 ppm (j) (compare Supporting Figure 1). The coupling of these signals is also clearly shown by a (d,j) cross peak in the COSY spectrum (see enlarged area in Figure 4).

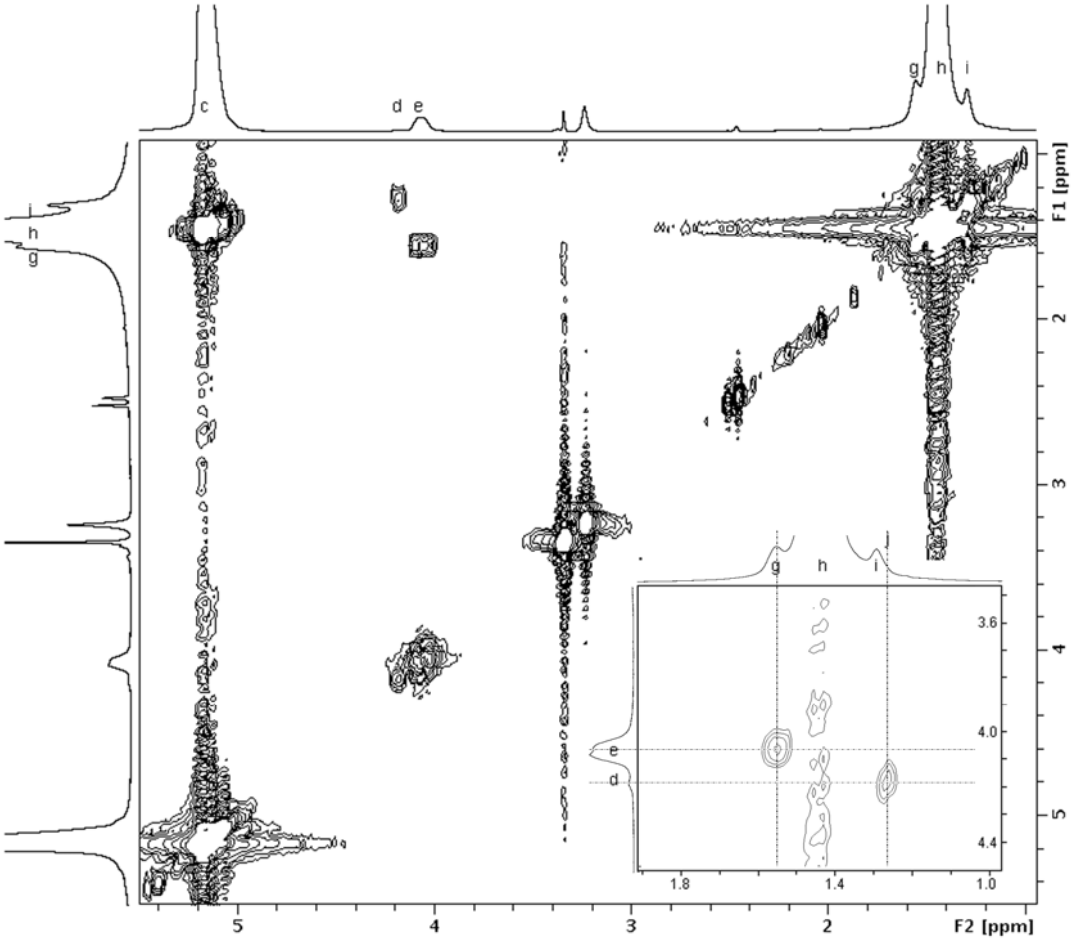

Figure 4. 2D HH-COSY NMR spectrum (HR-MAS) of the PDLLA network. The inserted magnification shows the coupling of the hexane peaks (e) and (g), and the coupling of the PI_p ethoxy methylene peak (d) to its neighboring methyl peak (j). Note, the peaks e, g and i have the same intensity (all hexane peaks), whilst d and j are much less intense and the latter is barely visible in the 1D spectrum. However, the d-j cross peak is evident.

Comparison of the integrals of the aromatic peaks in the PDLLA network spectra reveals a ratio of 70±4 % PI_p-initiated polymethacrylate chains in the network to 30±4 % PIa-initiated chains. In the photo-polymerization of styrene using a very similar photo-initiator (2,4,6-trimethylbenzoyl)diphenylphosphine oxide (TPO), the phosphinate radical exclusively initiates polymerization.[34] In our system, however, the PI_a species is also found to initiate radical polymerization of the methacrylate macromer end-groups. This observation was confirmed by photo-polymerization of methyl methacrylate initiated by Lucirin TPO-L. The resulting poly(methyl methacrylate) polymer could be characterized in solution by NMR, which clearly indicated the presence of both initiating species in the polymer (spectrum not shown).

Having assigned all proton signals in the network, the most informative region regarding determination of the kinetic chain length appears around 4 ppm. Here, the signal of the hexane methylene group next to a PDLLA-ester bond is present (e), just upfield by 0.1 ppm from the ethoxy-methylene of the photo-initiator unit PI_p (d). These peaks are partially overlapping but can be separated by deconvolution, enabling quantitative comparison (Figure 5). The two larger peaks are equally intense and correspond to the diastereotopic protons of the hexane methylene unit, neighboring the two enantiomeric lactic acid groups that are statistically equally abundant in the sample. The downfield PI_p peak is a single broad peak, about 40 Hz wide at half height, due to spin-spin coupling to the methyl group as well as to the ^{31}P nucleus. The average kinetic chain length (or degree of polymerization) is defined as the average ratio of monomers per chain initiator. We cannot quantitatively distinguish the methacrylate monomer peaks in our spectra. However, as each hexane methylene (hexanediol residue peak e, 2 protons) that connects to a lactide ester corresponds to one methacrylate monomeric unit in the kinetic chain (also 2 protons), the ratio of this hexane methylene peak to the PI_p peak allows direct estimation of the average kinetic chain length. This ratio is 13±3, and would correspond to the average kinetic chain length in case only PI_p initiator residue units would be present at the chain ends. Taking into account that the ratio of PI_p to PI_a chain ends is 70 % to 30 %, we can calculate an average ratio of

70 % x 13±3 = 9±2 methacrylate units per PI initiator-residue. This value represents the average kinetic PMA chain length, assuming no chain transfer took place. The number-average degree of polymerization $\overline{X}_n$ of the polymethacrylate chains depends on the mechanism of termination in the radical crosslinking reaction. Here, $\overline{X}_n$ is in the range of 9±2 to 18±4, in the extreme cases of termination by disproportionation only or by combination only, respectively.

In the resin the molar ratio of methacrylate groups to photo-initiator was 3.5, which implies a theoretical minimal average kinetic chain length of 1.75, assuming no chain transfer takes place. As we observe an average kinetic chain length of 9±2, this means that only between 21 and 33 % of the PI present in the resin is actually involved in the photo-initiated chain-crosslinking process (assuming that chain transfer does not occur). Nevertheless, relatively short kinetic chains are formed compared to other studies on methacrylate-crosslinked networks.[22, 23]

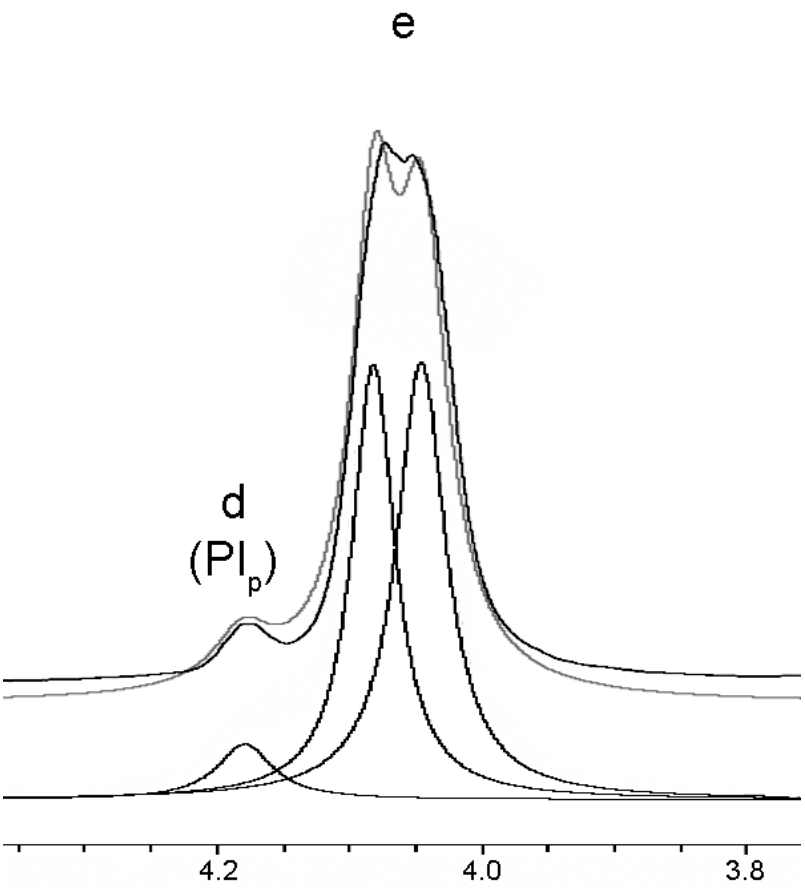

Figure 5. Example of peak deconvolution in ^{1}H-NMR HR-MAS spectrum. The measured spectrum (upper black peaks) is deconvoluted into the PI_p ethoxy-peak, and the peaks that correspond to the hexane methylene of the hexanediol residue that connects to a PDLLA chain. The sum of the deconvoluted peaks is shown in grey.

The kinetic chain length strongly depends on photo-crosslinking conditions such as photo-initiator concentration and light intensity. In the layer-by-layer fabrication process that takes place in a stereolithography apparatus, a dye is often used to limit the penetration of light into the resin. Besides the network films, of which the characterization is described above, we also prepared porous structures by stereolithography.[27] For this we used the same resin as for the preparation of the network films, but added 0.15 wt% orange dye. The average kinetic chain length was found to be 24±4 for these network structures. The attenuation of light by the dye causes a lower average light intensity, hence lower initiation rates, lower concentrations of propagating chains and eventually, longer chains. Another interesting difference observed between bulk photo-crosslinked networks and stereolithography-fabricated structures is the presence of unreacted methacrylate groups in the latter (5 to 15 %). These unreacted groups could either be further polymerized by post-curing, or they remain available for subsequent covalent coupling, for example to adjust the hydrophilicity of the networks or to immobilize cell-adhesive peptides at the surfaces of the porous structures.[35]

The PDLLA networks were degraded by refluxing in sodium hydroxide solution for 3 weeks, following removal of lactic acid, hexanediol and salts by dialysis. In the NMR spectrum of the degradation product depicted in Figure 6, the methacrylate protons are represented by the broad peaks around 1.8 (k') and 1.0 ppm (l'). The characteristic peaks of the PI-residues are not observed in the spectrum, so the average kinetic chain length could not be determined from the NMR spectrum of the degradation product. Other small peaks are observed that were not present in the network spectrum, and could not be assigned by COSY, NOESY, HMBC or HMQC experiments. Strikingly, lactic acid peaks are still clearly present at 4.8 (c) and 1.4 ppm (h). The molar ratio of methacrylate to lactic acid is

approximately one to one, which seems to imply that the lactic acid unit remains attached to the polymethacrylate backbone under the degradation conditions used. This would mean that the (PMA-LA) linking ester bond is much less prone to hydrolysis than the ester bonds within the polylactide chain. The insensitivity of methacrylate ester bonds to (alkaline) hydrolysis was observed earlier also for poly(ethylene glycol)dimethacrylate hydrogels and for poly(methyl methacrylate).[36] Taking into account the pendant lactic acid groups, and using the determined value of $\overline{X}_n$ of 9±2 to18±4 units, we can calculate an average molecular weight of the poly(lactic acid methacrylate) chains after degradation of 1.1-3.5 kg/mol for the photo-polymerized PDLLA networks. For the porous PDLLA network structures that were prepared by stereolithography using the dye-containing resin, the molecular weight of the degradation product will be 3.2-8.8 kg/mol assuming no chain transfer took place. In these ranges of molecular weights, renal clearance is still effective and fast, so no accumulation of degradation product is expected.

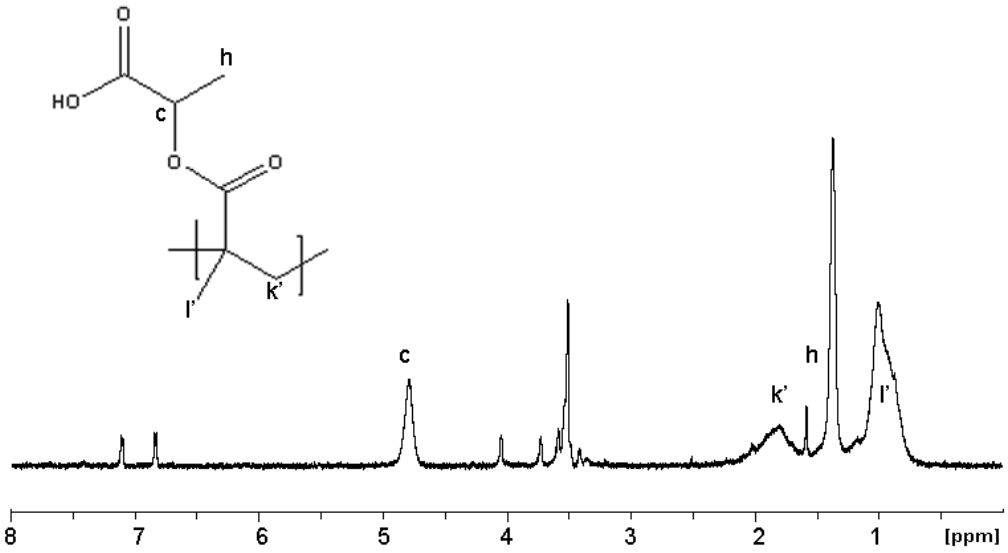

Figure 6. ^{1}H-NMR spectrum of the degradation product of photo-crosslinked PDLLA network in DMSO-d_6, after filtering out water and DMSO by a diffusion filter. The structure represents the repeating unit of the poly(lactic acid methacrylate) chains. The labels correspond to those in the network structure (Figure 2).

Degradation kinetics of networks

These biodegradable PDLLA networks are suitable materials for biomedical applications such as orthopedics and (bone) tissue engineering. They have favorable mechanical and physical properties, and can easily be prepared in any shape using stereolithography. Furthermore, we have seen that the kinetic chain lengths of the networks enable renal clearance of their degradation products. The time that it takes to degrade the networks is of utmost importance for their application. Photo-crosslinked PDLLA specimens were incubated at 37 °C in PBS to study the development of network properties in time. The degree of swelling in the solvent NMP was measured, as well as the gel content (Figure 7). These properties can give an indication of the extent of main chain scission before mass loss sets in. Both properties remained practically unchanged for the first 13 weeks of immersion in PBS. Due to PDLLA chain scission the degree of swelling starts to increase from around week 13, followed by a decrease in gel content starting from week 17. At this stage, dangling PDLLA chains that are cleaved at a second ester bond are removed from the network by solvent extraction. After 23 weeks, solvent-swollen networks were too fragile to be weighed so the degree of swelling could no longer be determined.

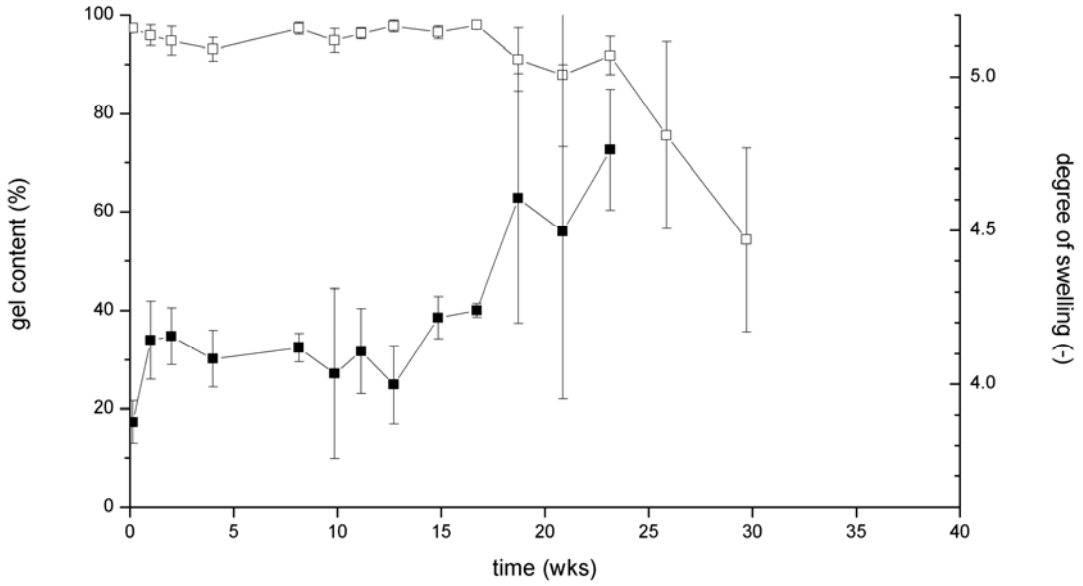

Figure 7. Network properties of photo-crosslinked PDLLA degraded in PBS at 37 °C: gel content (open squares) and degree of swelling in N-methyl-pyrrolidone (black squares). Each data-point represents average ± standard deviation of 3 specimens.

Degradation of biodegradable implants such as tissue engineering scaffolds is often expressed in terms of decrease in molecular weight and mass loss. However, the period that a medical implant is functional depends more on the preservation of its mechanical stability, which is usually much shorter. Figure 8 shows the development of the mechanical properties of photo-crosslinked PDLLA networks in time, together with the water uptake and mass loss. Here, the initial tensile strength was 47±2 MPa with an elongation at break of 2.3±0.3 %. Photo-crosslinked PDLLA retains its strength for a long time, compared to its mass loss profile. The strength remains practically unchanged up to 15 weeks (corresponding to the period that the network properties remain unchanged as well (Figure 7)) and it takes about 22 weeks -or 5 months- before half of its strength is lost. After loss of mechanical stability at week 24 (after which specimens become too fragile to be subjected to tensile testing), it takes only approximately 3 months before the network collapses and almost all mass is lost in a very short period. This degradation profile, in which the strength is retained for a long period compared to the mass loss, makes these networks very well suited for the intended application.

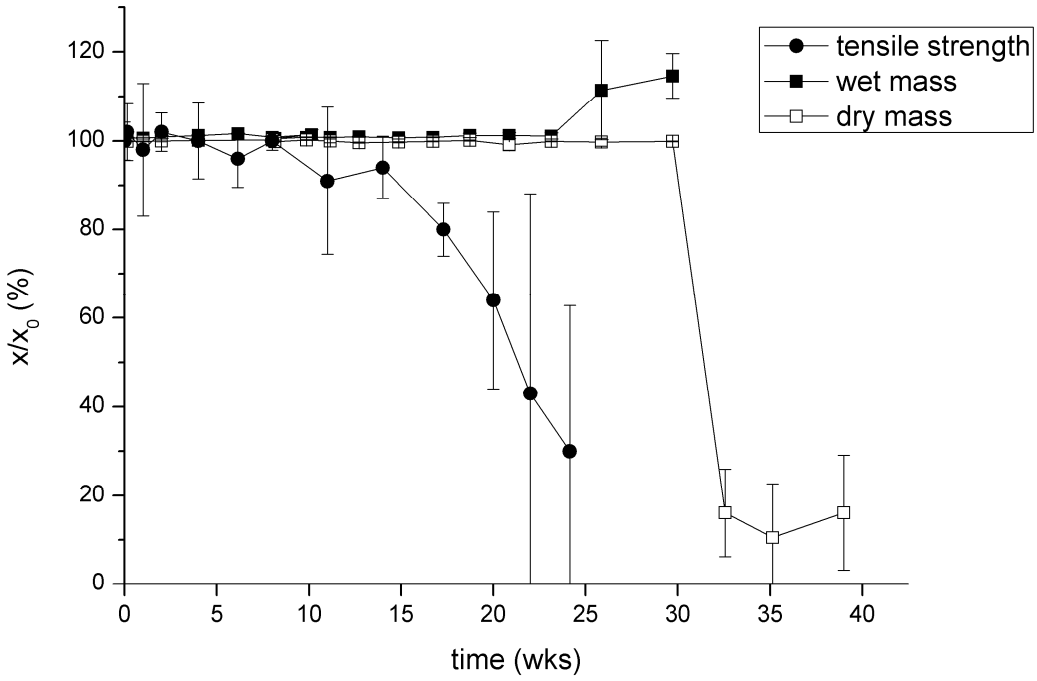

Figure 8. Properties of photo-crosslinked PDLLA networks degraded in PBS at 37 °C. Each data-point represents average ± standard deviation of 3 specimens.

High molecular weight PDLLA (HMW PDLLA) was included in this degradation study as a reference material. As the presence of residual monomer strongly influences the degradation behavior (decay in mechanical properties in particular),[19] it should be noted that this commercially available HMW PDLLA contained 1.7±0.4 % monomer (determined by ^{1}H-NMR). Although the mass loss profile is similar to the PDLLA networks, the decay in tensile strength differs greatly (Figure 9). Initially the tensile strengths of HMW PDLLA (50±2 MPa) and photo-crosslinked PDLLA (47±2 MPa) are comparable, but directly after immersion in PBS the strength of the linear polymer decreases rapidly. After only 1 month it has lost most of its structural integrity and strength. The decrease of the intrinsic viscosity in time (initial value 2.38±0.06 g/dL) is similar to that of the tensile strength, indicating high chain scission rates in the polymer. Furthermore, HMW PDLLA shows an undesirably high water uptake of up to more than 100 % after 20 weeks, while the PDLLA networks increase in weight only with a few percent. In the networks, it is likely that the crosslinks prevent extensive swelling in water and specimens retain their shape. The observations lead to the conclusion that the presence of crosslinks

has little effect on the course of hydrolysis reactions, but strongly influences the geometrical and mechanical stability of the degrading specimens.

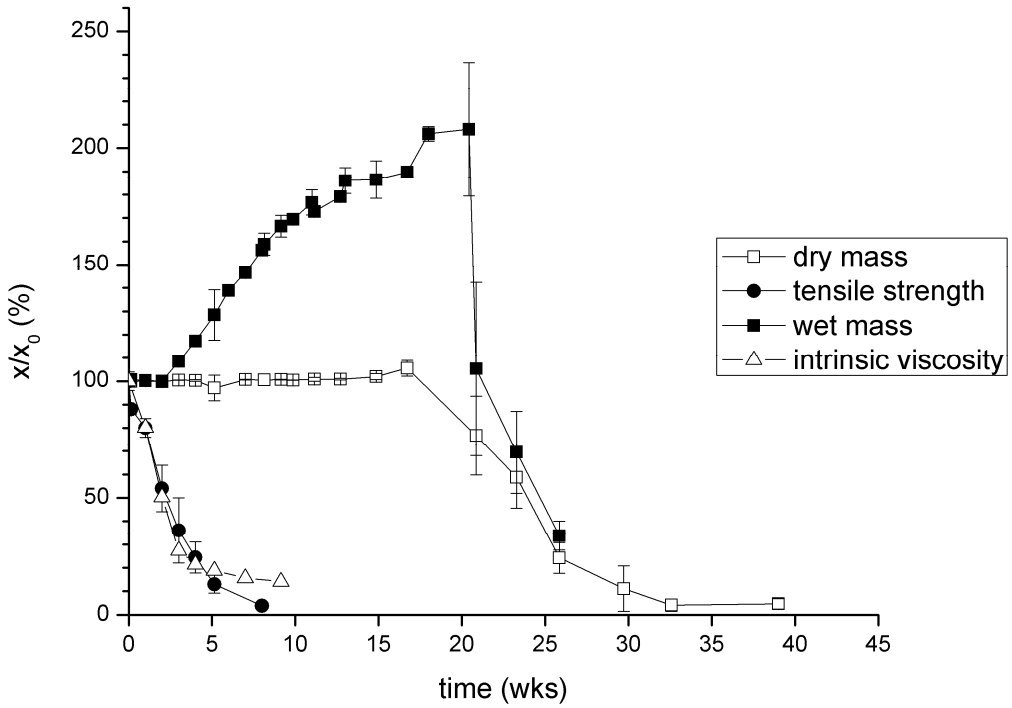

Figure 9. Properties of HMW PDLLA degraded in PBS at 37 °C. Each data-point represents average ± standard deviation of 3 specimens, except for the intrinsic viscosity (n=1).

Semi-crystalline poly(L-lactide) (PLLA) is often employed for medical applications rather than amorphous PDLLA. Although longer-term mechanical stability is achieved as the degradation is much slower in the crystalline phase, still the strength is lost long before mass loss sets in. Moreover, upon degradation of the amorphous phase PLLA releases crystallites, which can remain in the body for several years and induce foreign body reactions and swelling.[37] For these reasons, amorphous PDLLA networks form a good alternative to both amorphous PDLLA as well as crystalline PLLA. The chemical crosslinks preserve mechanical stability, while the degradation period after loss of strength is short and no potentially harmful crystalline regions are present.

Conclusions

The network structure and hydrolytic degradation of poly(D,L-lactide) (PDLLA) networks, photo-crosslinked from PDLLA-dimethacrylate oligomers, was studied. NMR analysis of the degradation products in solution revealed the presence of one lactic acid unit per methacrylate unit on the polyaddition chains, but could not be used to determine kinetic chain lengths. High-resolution NMR spectroscopy under magic angle spinning conditions (HR-MAS) proved a very useful and sensitive technique to characterize such polymer networks in the solvent-swollen state. Identification of all network components was achieved, and an average kinetic chain length of 9±2 could be determined. This implies an average molecular weight of the poly(lactide methacrylate) chains after degradation in the range of 1.1-3.5 kg/mol. For computer-designed PDLLA network structures prepared by stereolithography in the presence of a dye, the average molecular weights of these polyaddition chains are 3.2-8.8 kg/mol. These values are far below the threshold for renal clearance.

When incubated at 37 °C in PBS, the PDLLA networks were shape-stable and showed little mass change for approximately 6 months, shortly followed by complete collapse and near-complete mass loss. For the first 15 weeks, tensile strength, gel content and degree of swelling remained practically unchanged. After this time point, chain scission was evidenced by a reduction in strength and gel content, and by an increase in degree of swelling (implying a decrease in crosslink density).

The characterized PDLLA networks show excellent mechanical properties over a period of approximately 5 months in aqueous environments, and they degrade into water-soluble products with molecular weights far below the kidney cut-off. Therefore, these networks hold great promise for orthopedic and (bone) tissue engineering applications.

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SUPPORTING INFORMATION AVAILABLE

^{1}H-NMR spectrum of the photo-initiator and NOESY-spectrum of the PDLLA network. This information is available free of charge via the Internet at http://pubs.acs.org/.

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